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HYDROQUINONE: A GENERAL PHAGOSTIMULATING PHEROMONE IN TERMITES

JUDITH REINHARD,[1,*] MICHAEL J. LACEY,[1] FERNANDO IBARRA,[2] FRANK C. SCHROEDER,[3] MANFRED KAIB,[4] and MICHAEL LENZ[1]

[1]*CSIRO Entomology*
GPO Box 1700, Canberra ACT 2601, Australia

[2]*Institut für Organische Chemie*
Universität Hamburg
20146 Hamburg, Germany

[3]*Department of Chemistry and Chemical Biology*
Cornell University
Ithaca, New York 14853
USA

[4]*Lehrstuhl für Tierphysiologie*
Universität Bayreuth
95440 Bayreuth, Germany

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Abstract—The organization of termite societies depends predominantly on intraspecific chemical signals (pheromones) produced by exocrine glands, which induce and modulate individual behavioral responses. Here, the saliva-producing labial glands of termites were investigated with respect to their pheromonal role in communal food exploitation of termite colonies. From these glands, we identified for the first time hydroquinone (1,4-dihydroxybenzene) as a phagostimulating pheromone in the Australian termite species *Mastotermes darwiniensis*. Hydroquinone is released from the labial glands of termite workers and applied onto the food. It stimulates nestmates to feed at the spot of application and is, thus, employed to mark feeding sites. No synergistic effect with other identified labial gland compounds, such as glucose, inositol, and arbutin, was evident. Significantly, we show that termite species from all over the world, irrespective of taxonomic position and biological traits, produce and employ hydroquinone as phagostimulating signal. The use of the same chemical signal throughout an order is a unique phenomenon, not reported before in animals. Its possible biosynthetic pathway, ecological significance, and evolution are discussed.

*To whom correspondence should be addressed at current address: Judith Reinhard, c/o Termite Research Group, CSIRO Entomology, GPO Box 1700, Canberra Act 2601, Australia. E-mail: judith1212@hotmail.com

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Key Words—Termites, pheromones, feeding stimulation, labial glands, hydroquinone, arbutin.

INTRODUCTION

Termites are social insects living in highly organized communities of up to several million individuals. Communication among nestmates is essential for the coordinated functioning of a termite colony (Pasteels and Bordereau, 1998). This is achieved predominantly by intraspecific chemical signals (pheromones) produced by exocrine glands, which induce and modulate individual behavioral responses. Compounds from sternal and frontal glands regulating foraging, recruitment, alarm, and defense are well investigated (Pasteels and Bordereau, 1998; Kaib, 1999). The labial glands of termites have received only minor attention regarding the chemistry of their secretion and possible pheromonal role. These glands are located in the thorax, and the glandular ducts open into the oral cavity (e.g., Costa-Leonardo and Cruz-Landim, 1991; Kaib and Ziesmann, 1992). The aqueous labial gland secretion (contributing to the saliva) is released onto the substrate. The secretion contains numerous compounds and has diverse nonpheromonal functions. It has been reported to be defensive (Moore, 1968; Maschwitz and Tho, 1974; Olagbemiro et al., 1988), to play a role in nest construction and regulation of nest climate, and to function as a fungicide, social nutrient, and source of digestive enzymes (Noirot, 1969; Hewitt et al., 1971; Martin and Martin, 1978; Grassé, 1982; Brian, 1983; Hogan et al ., 1988; Veivers et al., 1982, 1991; Mednikova, 1991).

Behavioral data from previous studies suggested a pheromonal role of the labial gland secretion in *Reticulitermes santonensis* (France) and *Schedorhinotermes lamanianus* (East Africa). It appeared to contain a polar, heat-resistant chemical signal (Kaib and Ziesmann, 1992; Reinhard and Kaib, 1995, 2001 a,b; Reinhard et al., 1997a), that is released onto the food by feeding termite workers and stimulates nestmates to feed at the same site, thereby initiating clusters of feeding termites. Both termite species belong to the same family, Rhinotermitidae, but they differ in many aspects of their biology, such as habitat, nesting habits, and foraging and recruitment strategies (Harris, 1968; Becker, 1970; Schedel and Kaib, 1987; Kaib and Brandl, 1992; Brandl et al., 1996, Reinhard et al., 1997b). Nevertheless, both species appear to produce a chemically similar, if not identical, phagostimulating pheromone in their labial glands. Further studies using bioassays combined with thin-layer chromatography indicated that numerous other termite species possess the phagostimulating signal as well (Reinhard and Kaib, 2001a,b). The work presented here was undertaken to identify the chemical nature of the phagostimulating pheromone from labial glands of termites.

METHODS AND MATERIALS

Insect Species. Most experiments were first carried out with the Australian termite species *Mastotermes darwiniensis. M. darwiniensis* were collected near Darwin, Australia, and maintained in the laboratory in groups of ca. 1000 individuals in plastic containers with water and wood at 30°C, 90% relative humidity for the bioassays. Other termite species (Table 2 below) were collected in Australia and Africa, or provided from the laboratory cultures of the Bundesanstalt für Materialforschung und -prüfung, Berlin, Germany, and the University of Bern, Switzerland. Cockroaches (*Periplaneta americana*) were taken from a laboratory culture of CSIRO Entomology, Australia.

Preparation of Samples. Labial gland extracts of termite workers and cockroaches were prepared by excising between 6 and 120 labial glands (depending on species size), without the gland reservoirs, as the latter do not contain the active signal (Kaib and Ziesmann, 1992; Reinhard et al., 1997a). Glands were extracted in distilled water (500 μl) for 12 hr at 4°C, then deep-frozen for 24 hr. Thereafter, the glands were removed. Saliva (100 μl) of *M. darwiniensis* was collected with glass microcapillaries from the oral cavity and extracted in distilled water (500 μl). For the gnawed filter paper extract, filter paper was offered to 50 *M. darwiniensis* workers in Petri dishes overnight, and the following morning excised pellets (corresponding to an area of ca. 5 cm^2) were extracted over glasswool in distilled water (1 ml). Extracts of clean filter paper were prepared as controls. All extracts were used for both feeding bioassays and chemical analyses. For bioassays with synthetic references, the compounds were dissolved in distilled water with or without the antioxidant BHT (2,6-bis(1,1-dimethylethyl)-4-methylphenol; butylated hydroxytoluene) added in 10% ratio. Solution series were prepared corresponding to the natural concentrations of the compounds in the labial glands of the respective species.

Bioassays. All biological experiments were conducted under red light illumination. Feeding behavior in termite workers can be recognized by the hypognathous head position and wriggling head movements while gnawing and tearing off food fibers (Reinhard and Kaib, 2001a,b). Feeding spots are recognized afterwards by marks or holes in the substrate due to removal of food particles by the feeding termites. Foraging arenas for the feeding choice tests were made of large Petri dishes (14.5 cm ID, 1 cm high). They were attached to the containers holding *M. darwiniensis*. In each test, two semicircles (2.5 cm diam.) of filter paper were offered, one treated with 25 μl extract (labial gland, saliva, gnawed filter paper extract) or a solution of synthetic chemicals, the other moistened with 25 μl distilled water (control). For each bioassay, the spatial distribution of the first 20 termites feeding on the two semicircles according to the behavior described above was determined during the test duration of ca. 1 hr. Termites arresting or aggregating without actually feeding were not considered. Tests were

replicated 20 times and analyzed with the sign test on an ordinal level of the data (Zar, 1974). For the other termite species tested, the bioassay set-up was modified with respect to size of nest containers and arenas, observation method (direct or recorded by IR-sensitive video techniques), and duration of tests. Tests in the field were conducted with colonies of *C. curvignathus* in Malaysia, using paper towels (10 × 40 cm) treated with 40 μg hydroquinone and untreated ones as control, offered in open plastic containers for 24 hr. The results were photographed.

Chemical Analysis. Chemical analyses were based on GC-MS investigation, with a Hewlett Packard 5890 gas chromatograph coupled to VG Trio 2000 mass spectrometer. 1,2-Dihydroxyphenol (Aldrich) was added to the extracts described above as an internal standard for quantification of the pheromone. After the extracts were concentrated to 30 μl under nitrogen and freeze-dried, the residues were taken up in *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Alltech, 50 μl), and pyridine (15 μl) and silylated at 90°C for 1 hr. The samples (1 μl) were injected on-column (Alltech EC 5, 30 m long × 0.32 mm ID, film thickness 0.5 μm), with helium as the carrier gas (flow rate 1 ml/min). The GC was operated using the following temperature program: 90°C for 6 min, a gradient of 20°C/min to 180°C, followed by a gradient of 10°C/min to 300°C, and isothermal for 20 min at 300°C. The effluent was subjected to positive-ion chemical ionization with NH_3 as reactant gas, and the monitored ions for the pheromone and internal standard were $[M+NH_4]^+$ and $[M+NH_4-H_2O]^+$. For general quantification of the remaining labial gland components, apart from the pheromone, Tri-Sil (Pierce, 20 μl) was added to the above samples and heated at 90°C for further 3 hr to ensure complete silylation. These components were quantified by gas chromatography (GC Varian 3300, on-column injection, Alltech EC 5, temperature program 60°C for 1 min, followed by successive gradients of 50°C/min to 150°C and 10°C/min to 280°C isothermal for 10 min).

RESULTS

In feeding choice tests with laboratory colonies of *Mastotermes darwiniensis*, semicircles of filter paper, onto which we applied extracts of labial glands of *M. darwiniensis* workers or water as control, were offered as food. Labial gland extract clearly stimulated feeding in *M. darwiniensis* (Figure 1a). Saliva extracts and extracts of filter paper pellets that *M. darwiniensis* workers had previously chewed and salivated upon (gnawed filter paper) also stimulated feeding significantly. For the chemical analyses, we used persilylated samples of labial gland extracts, saliva, and gnawed filter paper. For the first time in *M. darwiniensis* workers, we identified hydroquinone (1,4-dihydroxybenzene) in small amounts

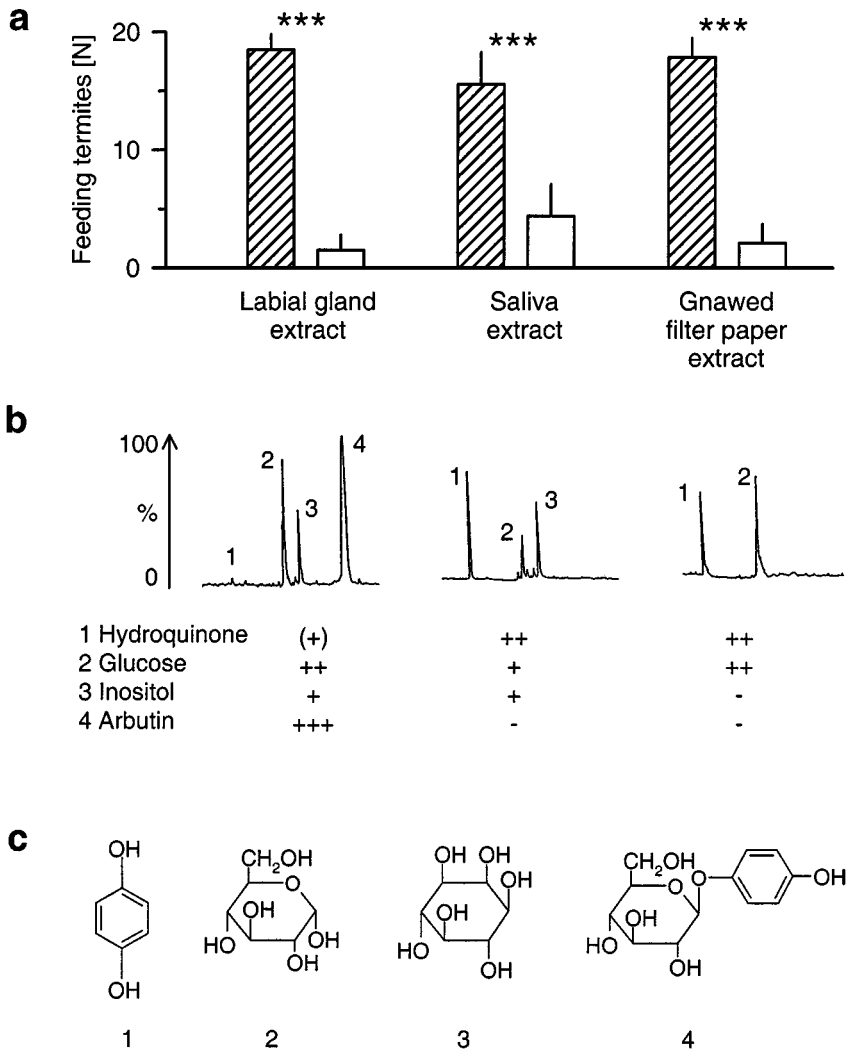


FIG. 1. Phagostimulating effect and chemical analysis of *Mastotermes darwiniensis* extracts. (**a**) Feeding choice tests with labial gland, saliva, and gnawed filter paper extract (25 μl extract per test). Mean distribution ($\pm$SD, $N = 20$) of the first 20 termites feeding on two semicircles of filter paper; hatched bars: semicircle treated with extract, empty bars: semicircle moistened with water as control. ***: significant difference at $P < 0.001$, sign-test. (**b**) Gas chromatograms of the above extracts (y axis: relative abundance) and ratio of identified compounds. $+++$: major component, $++/+$: minor components, $(+)$ trace component, $-$: not present. (**c**) Chemical structures of identified compounds.

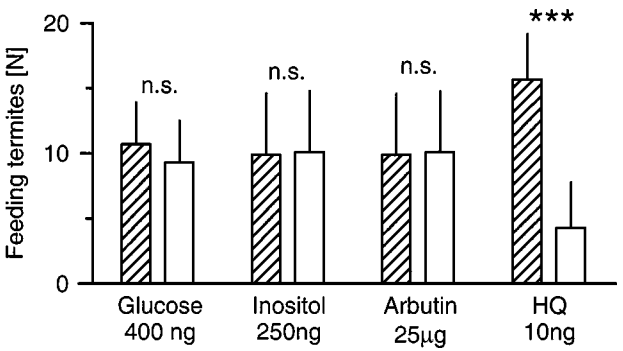


FIG. 2. Phagostimulating effect of labial gland components of *Mastotermes darwiniensis*. Compounds tested in naturally extractable concentrations (amount per labial gland) in a feeding choice bioassay. Mean distribution ($\pm$SD, $N = 20$) of the first 20 termites feeding on two semicircles of filter paper; hatched bars: semicircle treated with compound, empty bars: semicircle moistened with water as control. n.s.: no significant difference, ***: significant difference at $P < 0.001$, sign-test.

by gas chromatography–mass spectrometry (GC-MS) analysis of the labial gland extract (Figures 1b,c). We also identified glucose, an inositol, and arbutin (4-hydroxyphenyl-β-glucopyranoside) in labial gland extracts, corresponding to earlier preliminary results (Reinhard, 1998). The saliva and gnawed filter paper extracts contained glucose and an inositol, or glucose only, respectively. Arbutin was not detectable in the latter extracts, but hydroquinone was found instead as a major component (up to 0.64 μg/μl saliva). When each of the identified components was assessed individually at natural concentrations in the feeding choice test, only hydroquinone stimulated feeding in *M. darwiniensis* (Figure 2), corresponding to the activity of saliva and labial gland extracts. Mixtures of hydroquinone with other gland components were tested for phagostimulation, but a synergistic effect was not evident (Table 1). Thus, it was concluded that hydroquinone is the phagostimulating pheromone derived from the labial glands.

Tests with a concentration series of hydroquinone, from 1 ng to 10 μg per test, revealed that for *M. darwiniensis* it was effective as a phagostimulant only at 5–10 ng (Figure 3). At higher concentrations, the effect was diminished. However, when the synthetic antioxidant BHT was added in 10% ratio to hydroquinone, the phagostimulant proved to be effective at concentrations up to 10^3 times higher.

After analyzing the labial glands of a further 14 termite species from all major termite families collected throughout the world, we found that they all contained the same components: glucose, one or more inositols, arbutin, and a trace amount of hydroquinone (Table 2). On the other hand, labial glands of the cockroach

TABLE 1. PHAGOSTIMULATING EFFECT OF COMBINATIONS OF LABIAL GLAND COMPONENTS OF *Mastotermes darwiniensis*[a]

Compound(s)	Termites (N) on treated semicircle	Termites (N) on control semicircle	P^b
HQ	15.7 ± 3.5	4.3 ± 3.5	$P < 0.001$
HQ, G	15.4 ± 2.7	4.6 ± 2.7	$P < 0.001$
HQ, I	15.3 ± 3.6	4.7 ± 3.6	$P < 0.001$
HQ, A	14.1 ± 3.9	5.9 ± 3.9	$P < 0.01$
HQ, G, I	15.2 ± 3.4	4.8 ± 3.4	$P < 0.001$
HQ, G, A	14.6 ± 3.8	5.4 ± 3.8	$P < 0.001$
HQ, I, A	14.2 ± 3.8	5.8 ± 3.8	$P < 0.01$
HQ, G, I, A	15.1 ± 3.3	4.9 ± 3.3	$P < 0.001$

[a] HQ: hydroquinone 10 ng, G: glucose 400 ng, I: inositol 250 ng, A: arbutin 25 μg. Compounds tested in naturally extractable concentrations (amount per labial gland) in a feeding choice bioassay. Mean distribution ($\pm$SD, $N = 20$) of the first 20 termites feeding on two semicircles of filter paper; semicircle treated with compound(s) versus semicircle moistened with water as control.
[b] Significant difference, sign-test.

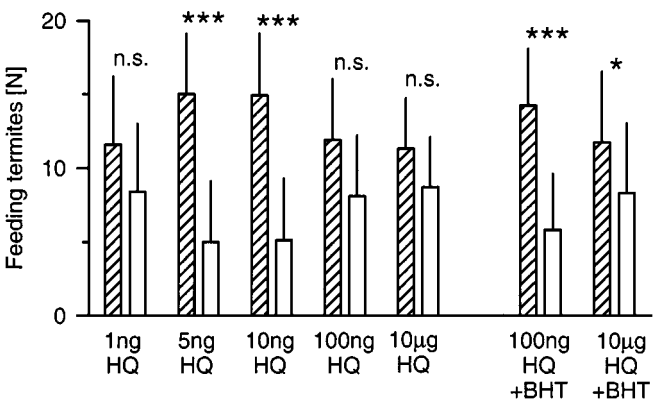


FIG. 3. Phagostimulating effect of different concentrations of hydroquinone (HQ) and mixtures of hydroquinone and the synthetic antioxidant butylated hydroxytoluene (BHT) added in 10% ratio, on *Mastotermes darwiniensis*. Mean distribution ($\pm$SD, $N = 20$) of the first 20 termites feeding on two semicircles of filter paper; hatched bars: semicircle treated with compound(s), empty bars: semicircle moistened with water as control. (n.s.: no significant difference, ***: significant difference at $P < 0.001$; *: significant difference at $P < 0.05$, sign-test).

TABLE 2. TERMITE AND COCKROACH LABIAL GLAND COMPONENTS[a]

No.	Family	Species	G	I	A	HQ
Termites						
1	Kalotermitidae	*Kalotermes flavicollis* (Italy)	83.6	170.8	296.7	0.02
2		*Cryptotermes brevis* (Australia)	13.2	15.9	17.0	5.10
3	Mastotermitidae	*Mastotermes darwiniensis* (Australia)	396.2	235.8	28827.9	10.00
4	Hodotermitidae	*Hodotermes mossambicus* (Kenya)	1128.0	595.2	255.9	0.12
5	Termopsidae	*Zootermopsis angusticolllis* (USA)	723.4	393.3	1891.9	0.73
6	Rhinotermitidae	*Reticulitermes flavipes* (USA)	27.6	93.6	44.1	0.05
7		*Reticulitermes santonensis* (France)	71.0	65.0	24.8	0.44
8		*Heterotermes indicola* (India)	20.1	42.5	10.2	0.02
9		*Schedorhinotermes lamanianus* (Kenya)	162.4	282.9	41.5	0.01
10		*Coptotermes formosanus* (Southeast Asia)	25.3	141.4	24.0	0.03
11		*Coptotermes acinaciformis* (Australia)	20.0	610.0	11.0	0.03
12	Termitidae	*Nasutitermes nigriceps* (Central America)	368.0	172.8	44.7	0.03
13		*Nasutitermes exitiosus* (Australia)	15.6	225.0	10.6	0.03
14		*Trinervitermes trinervoides* (South Africa)	123.0	51.6	25.5	0.04
15		*Macrotermes subhyalinus* (West Africa)	561.5	594.0	32.4	3.01
Cockroach						
16	Blattellidae	*Periplaneta americana*	2134.5	1433.6	0	0

[a] Quantification of components with GC and GC-MS. G: glucose, I: inositol(s), A: arbutin, HQ: hydroquinone; mean concentration ($N = 3$) in nanograms per labial gland. Species-specific variation correlated with size of glands.

Periplaneta americana contained glucose and inositols, but lacked arbutin (or other glucosides) and hydroquinone. Laboratory feeding choice tests with selected termite species (numbers 2, 3, 6, 7, 9, 10, 11, 13 in Table 2), using 0.1–5 ng hydroquinone per test, as well as first field trials with a Malaysian species revealed that, without exception, hydroquinone works as a powerful phagostimulant (Table 2, Figure 4).

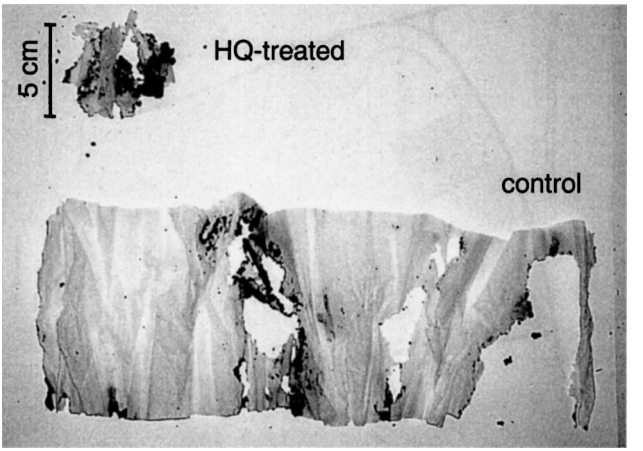


FIG. 4. Phagostimulating effect of hydroquinone on *Coptotermes curvignathus* (Malaysia) in the field. Exemplary trial showing remains of two paper towels (original size: 10 × 40 cm) that had been folded, placed into perforated plastic containers, and offered simultaneously as food to a termite colony for 24 hr. Above: paper treated with 40 μg hydroquinone (HQ), below: untreated paper (control).

DISCUSSION

Feeding bioassays with the Australian termite species *M. darwiniensis* demonstrated that a phagostimulatory signal, such as suggested for *R. santonensis* and *S. lamanianus* (Kaib and Ziesmann, 1992; Reinhard and Kaib, 1995; Reinhard et al., 1997a), also existed in the labial glands of *M. darwiniensis*. Subsequent chemical analyses and feeding bioassays identified hydroquinone as the phagostimulating pheromone from termite labial glands, even though present in only minute amounts. Verification of solubility, heat resistance, and TLC analyses of hydroquinone proved that its physicochemical properties corresponded to those suggested for the phagostimulant in previous studies (Kaib and Ziesmann, 1992; Reinhard et al., 1997a; Reinhard and Kaib, 2001a). Laboratory and field experiments evaluating quantitative data of food consumption in choice tests over a longer period of time have confirmed the phagostimulatory nature of hydroquinone (Reinhard et al., in press).

Hydroquinone seems to be effective only in the nanogram range. Its diminished effect at higher concentrations is possibly due to the oxidative formation of larger quantities of quinone (1,4-cyclohexadienedione) in the bioassay, which is generally known as an effective repellent and in some termite species is released as a defensive compound by soldiers (Moore, 1968; Olagbemiro et al., 1988). This assumption is supported by the fact that hydroquinone proved to be effective

at levels up to 10^3 times higher when the synthetic antioxidant BHT was added. Similar to other hydroquinone analogs tested, BHT alone does not have a significant phagostimulating effect (Reinhard et al., in press). Therefore, the increased effectiveness of hydroquinone in this experiment is indeed due to the antioxidative function of BHT.

The most remarkable aspect of the phagostimulating signal was revealed when the labial glands of termite species from all major termite families were analyzed. Independently of the phylogenetic classification, geographic origin, or living and feeding habits of these termite species, their labial glands contained hydroquinone as a phagostimulant. Interspecific sharing of single compounds within multicomponent pheromones occurs (Howard et al., 1976; Kaib et al., 1982; Borges et al., 1999), but the use of the same chemical signal throughout an order has not been reported before in animals. The labial glands of the cockroach *Periplaneta americana*, analyzed because cockroaches are assumed to be ancestors of termites (Kambhampati, 1995), lacked hydroquinone. The use of hydroquinone as phagostimulating pheromone seems to be a monophyletic trait of the Isoptera. Notably, however, the potency of hydroquinone as a phagostimulant for bark beetles, albeit at higher concentrations, has been known for some time (Norris, 1970, 1976).

This is the first report of the use of hydroquinone as a termite pheromone to organize communal food exploitation. As yet, we have not evaluated the amount of pheromone released by individual termites, nor do we know if other unidentified synergistic compounds or antioxidants are released simultaneously. Concerning the other compounds identified in the labial glands, the presence of inositols could be due to many factors, considering the numerous functions of inositols in cell biochemistry (Stryer, 1995; Berridge, 1997). Glucose and arbutin, on the other hand, may be associated with hydroquinone formation. While there is arbutin but little hydroquinone in the labial glands, there is significant hydroquinone and glucose but no arbutin in saliva and gnawed filter paper. Moreover, the termite diet consists mainly of carbohydrates (cellulose-derived) and aromatic compounds (lignin-derived), suggesting the following hypothetical pathway based on inducible enzymatic activities: termites acquire glucose, hydroquinone, and arbutin either directly in their food or via metabolism (Kreitsberg and Ekabsone, 1973; Noirot, 1992; Inoue et al., 1997; Oliver et al., 1998). Since hydroquinone itself is a slight irritant, and formation of the toxic quinone needs to be avoided, hydroquinone is stored as its stable conjugate arbutin, possibly synthesized by the enzyme phenol-β-glucosyltransferase (PGT) (Ahmad and Hokins, 1993). When required, arbutin can be readily broken down by the digestive enzyme β-glucosidase (Tokuda et al., 1997; Itakura et al., 1997), thus releasing hydroquinone. The trigger for β-glucosidase induction could be stimulants in the food and possibly hydroquinone itself, given the role of quinoid compounds in redox cycling, affecting cell signaling pathways (Brunmark and Cadenas, 1989; Schlatterer and Schaloske, 1996; Ambudkar, 2000).

The biological significance of the phagostimulating pheromone resides in the fact that food exploitation is a communal activity for all termite species. The phagostimulant promotes the formation of clusters of feeding termites, while successive application of saliva humidifies food and repeated biting enhances the mechanical break-up of its surface. In termite species that feed on dispersed food, such as the harvester termite *Hodotermes mossambicus*, the effect of the phagostimulant is not as obvious as in xylophagous termites, but nevertheless of importance. During the process of cutting long grass stems into pieces, the phagostimulant induces *H. mossambicus* workers to gather and bite into the same spot of the grass stem (Reinhard, 1998). Through such concentrated feeding efforts, a termite colony can exploit a food source faster and more efficiently.

It seems advantageous to use a simple chemical that is probably food-derived for such a frequently employed pheromone. Termites are herbivorous or humivorous, irrespective of the many different life and feeding traits within the group. All termites ingest similar compounds derived from cellulose and lignin, and they share basic digestive pathways (Abe, 1987; Noirot, 1992); therefore, food-derived signals are expected to be similar in all species. Furthermore, as termite species may share food sources, it can be advantageous to employ the same signal. Phagostimulation by hydroquinone appears to be an ancestral trait that has been strongly selected for in termites. We propose that this mechanism has evolved in concert with utilizing cellulose and lignin as a food source and has been developed into a strategy used by termites all over the world for organization of communal food exploitation.

Hydroquinone from termite labial gland secretion might also serve functions other than phagostimulation. For example, hydroquinone could be responsible for the fungicidal and aseptic effect demonstrated for the saliva of termites (Noirot, 1969; Grassé, 1982). Hydroquinone might also, serve a certain defensive role, acting as an irritant and repellent against intruders. It is notable that in termite species, in which the soldiers use the labial gland secretion for chemical defense, such as *Mastotermes*, *Odontotermes*, and *Macrotermes*, this secretion is composed of mixtures of benzo- and toluquinones (Moore, 1968; Maschwitz and Tho, 1974; Grassé, 1986; Olagbemiro et al., 1988). One could imagine that the production of quinones in the labial glands of the soldier caste has evolved out of the hydroquinone production in the worker caste.

These unanswered questions point to the most important consequence of the final identification of the phagostimulating pheromone in termites: our discovery opens up a whole range of new studies on function, biosynthesis, and evolution of hydroquinone, as well as on the application of the pheromone for termite control.

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EVIDENCE OF AN ASSEMBLY PHEROMONE IN THE BLACK-LEGGED DEER TICK, *Ixodes scapularis*

S. A. ALLAN[1,*] and D. E. SONENSHINE[2]

[1]*Department of Pathobiology, PO Box 110880*
University of Florida, Gainesville, Florida 32610-0880

[2]*Department of Biological Sciences*
Old Dominion University
Norfolk, Virginia 23529-0266

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Abstract— The responses of *Ixodes scapularis* (Acari: Ixodidae) nymphs and adults to extracts of cast larval skins were tested in a Petri dish bioassay. Assembly was elicited in nymphs and adults in the presence of skins, exudate from ticks, and filter paper exposed to ticks compared to untreated controls. Assembly was noted by 1 hr after exposure with little change between 1 and 24 hr. The assembly response increased in the presence of an increased number of skins. Similar assembly was elicited in nymphs and adults in the presence of cast larval skins and a saline (0.95% NaCl) skin extract. Methanol and hexane extracts were not attractive. When chemical standards were tested against nymphs, they responded to guanine, uric acid, hypoxanthine, xanthine, inosine, and hematin. Adults were tested against guanine, inosine, and xanthine, and all elicited significant assembly. Responses of nymphs increased significantly with increase in dose of uric acid and guanine. Responses of nymphs to a mixture of guanine, xanthine, and adenine (25:1:1 ratio) were similar to responses to cast skins. This study provides the first evidence of an assembly pheromone in *I. scapularis*.

Key Words—Assembly pheromone, tick, *Ixodes scapularis*, purines, guanine.

INTRODUCTION

Relatively little is known about the pheromonal regulation of behavior of the black-legged tick, *Ixodes scapularis* (Acari: Ixodidae) despite its public health importance as the primary vector of Lyme disease, human granulocytic ehrlichiosis, and human babesiosis in the United States (Spielman et al., 1985; Walker and

*To whom correspondence should be addressed. E-mail: sallan@gainesville.usda.ufl.edu

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Dumler, 1996; Childs et al., 1998). Reports of pheromones in other *Ixodes* species are also limited. Graf (1975) reported that a water-soluble pheromone produced by female *I. ricinus* strongly attracted males and, to a lesser degree, females. This pheromone appeared to facilitate mate finding and mating through an assembly response. Evidence of a water-soluble pheromone inducing assembly has also been documented for *Ixodes holocyclus* (Treverrow et al., 1977), *Ixodes persulcatus* (Uspensky and Emelyanova, 1980) and *I. ricinus* (Hájkova and Leahy, 1982), however, these pheromones are yet to be characterized or identified.

Assembly pheromones typically induce formation of off-host clusters of various stages and sexes of ticks through arrestment of movement upon contact. These assemblies are best described in soft ticks and tend to occur in caves, under ledges, and in cracks and crevices in proximity to nests of the hosts. These assemblies are thought to enhance mating and host-finding success (Sonenshine et al., 1982). Pheromone-induced assembly was first described from *Argas* ticks by Leahy et al. (1973). Subsequently, this behavior has been reported from at least 14 species of soft ticks as well as from several hard ticks including *Hyalomma dromedarii* (Leahy et al., 1981), *Rhipicephalus appendiculatus, Amblyomma cohaerans* (Otieno et al., 1985), *Rhipicephalus evertsi* (Gothe and Neitz, 1985), and the above mentioned species of *Ixodes*. Assembly pheromones are present in the excreta and the aqueous and saline washings of ticks (Leahy et al., 1973; Gothe and Kraiss, 1982). Best described from several species of *Argas*, assembly pheromones appear to consist of guanine and other purines such as xanthine and hypoxanthine that are present in extracts of nitrogenous excreta of these ticks (Neitz and Gothe, 1984; Otieno et al., 1985; Gothe, 1987; Dusbábek et al., 1991a,b). The existence of similar pheromones has not been previously reported from *Ixodes scapularis* and the objective of this study was to determine if assembly pheromones are present in *Ixodes scapularis*.

METHODS AND MATERIALS

Ticks. Ticks were obtained from a laboratory colony maintained at the University of Florida. Adult ticks were fed on New Zealand white rabbits and immatures fed on rats. Ticks were held in screen-topped vials at $24 \pm 1^{\circ}$C and 93–96% relative humidity under a 16L:8D photoperiod.

Bioassay Procedure. Responses of ticks were evaluated in Petri dish bioassays modified from Leahy et al. (1973). Disposable plastic Petri dishes (100 mm diam.) were placed over similar-sized paper templates divided into six equal-sized sectors. Sectors of the templates were labeled from 1 to 6. Teflon discs of 1 cm diam. (Thomas Scientific, Swedesboro, New Jersey) were treated with treatment solutions or solvent (control) and placed in the center of sector 1. None of the other sectors in the Petri dish received a treatment or control disc. Treatments or

controls were placed on Teflon discs and the solvent allowed to dry before being placed in the Petri dish. After the disc was added to the dish, 10 ticks were added in the center of the dish and the dish placed in a high-humidity (>93% relative humidity) chamber. The location of ticks by sector was noted at 1, 2, and 24 hr after placement to evaluate the cumulative effect of the assembly. Initial bioassays determined that there was no location bias under these experimental conditions and that there was no significant difference in numbers of ticks present in any sector (data not presented).

Bioassays. Initial bioassays were conducted using cast skins collected from blood-fed larvae, filter paper exposed to blood-fed larvae, and the exudate from blood-fed larvae. Skins shed by emerged nymphs were collected from tick rearing vials within a week after being shed. Skins were collected using fine-tipped forceps or a paintbrush, and only whole skins were used for extracts. Filter paper strips (1 × 4 cm) were placed in tick rearing vials along with 10 freshly blood-fed larvae. After 30 days of exposure, filter strips were removed from the vials and evaluated in bioassays. Exudate from the sides of glass tick rearing vials was removed by scraping with forceps and collected for use in bioassays. Bioassays were conducted immediately after collection of skins, filter paper, or exudate. Bioassays were conducted by placing cast skins (10 or 25), folded contaminated filter paper strips, or exudate (0.5 mg) on a Teflon disc in the center of sector 1. Controls consisted of an untreated Teflon disc or clean filter paper.

Extracts of cast skins were made using solvents covering a range of polarities and bioassay responses compared to those using cast skins. Cast skins were placed in 500 μl of 0.95% NaCl, vortexed three times for 20 sec, allowed to sit for 10 min, and then all liquid was transferred to a separate vial. Skins were washed twice times with 100 μl of 0.95% NaCl, and washes were added to the original extract and reduced under N_2 to a concentration of 1 skin equivalent/μl. Similarly, skins were washed with methanol (HPLC grade) and the extract reduced to 1 skin equivalent/μl. An extract with hexane (HPLC grade) was also prepared following the protocol for the above extracts. Bioassays were conducted comparing responses of *I. scapularis* females, males, and nymphs to cast larval skins, saline, methanol, and hexane extracts and untreated controls. Treatments were tested at 10 and 20 skins or skin equivalents. All bioassays were replicated 15 times.

Responses of *I. scapularis* were tested to a series of compounds previously reported as potential components of an assembly pheromone in soft ticks (Dusbábek et al., 1991a,b). Compounds tested included guanine, uric acid, hypoxanthine, xanthine, inosine, adenine, and hematin (Sigma, St. Louis, Missouri). Compounds were diluted in 0.95% NaCl and tested at 0.5 mg, which was a dose eliciting response in the study by Dusbábek et al. (1991a). Controls consisted of 1 μl of 0.95% NaCl. Prior to bioassays, Teflon discs were treated with 0.5 mg of each compound,

discs were allowed to air dry, and then bioassays were conducted. Bioassays were replicated 10 times for nymphs and 15 times for adults.

Compounds eliciting significant responses were tested over a range of doses to examine dose–response relationships. Responses of *I. scapularis* nymphs to 0.05, 0.5, and 5 mg of guanine, uric acid, xanthine, and adenine were examined in Petri dish bioassays and replicated 10 times.

Mixtures of purines consisting of guanine, xanthine, and adenine in a 25:1:1 ratio elicited responses in *A. persicus* equivalent to the natural pheromone (Dusbábek et al., 1991a). Comparisons were made of responses of *I. scapularis* nymphs to concentrations of this purine mixture ranging from 10 to 0.001 mg.

Analysis. Data were transformed [log $(x + 1)$] prior to statistical analysis; however, original numbers are presented in tables and figures. Data were analyzed by ANOVA and differences between means located by paired *t* test.

RESULTS

Cast larval skins, tick-exposed filter paper, and tick exudate elicited more assembly response in nymphs than untreated controls (Table 1). Within 1 hr, there were more ticks in sectors with larval skins ($t = -5.93$, $df = 18$, $P < 0.001$), exposed filter paper ($t = -9.76$, $df = 18$, $P < 0.001$), and exudate ($t = -5.69$, $df = 18$, $P < 0.001$) compared to untreated controls. Similarly by 2 and 24 hr, there were more ticks in the sectors with larval skins ($t = -3.99$, $df = 18$, $P < 0.001$; $t = -4.8$, $df = 18$, $P < 0.001$, respectively), exposed filter paper ($t = -11.4$, $df = 18$, $P < 0.001$; $t = -13.5$, $df = 18$, $P < 0.001$, respectively), and exudate ($t = -6.39$, $df = 18$, $P < 0.001$; $t = -5.3$, $df = 18$, $P < 0.001$, respectively) than in the controls. There was no difference in tick response between 1 and 24 hr in Petri dishes containing larval skins ($t = -0.48$, $df = 18$, $P = 0.31$), exposed filter paper ($t = 0$, $df = 18$, $P = 0.5$), exudate ($t = 1.32$, $df = 18$, $P = 0.1$), or untreated controls ($t = 1.34$, $df = 18$, $P = 0.09$).

TABLE 1. ASSEMBLY OF *I. scapularis* NYMPHS IN PETRI DISH SECTORS[a]

	Ticks in treatment sector (zone 1) (%, mean ± SE)			
Time (hr)	Untreated control	Cast skins	Exposed filter paper	Exudate
1	8.0 (2.5)	36.1 (4.0)[b]	40.8 (2.1)[b]	30.0 (2.9)[b]
2	4.0 (1.6)	34.2 (7.3)[b]	38.5 (2.5)[b]	24.2 (2.6)[b]
24	4.0 (1.6)	40.0 (7.3)[b]	40.2 (2.1)[b]	24.0 (3.4)[b]

[a] Petri dishes contained cast larval skins (10), filter paper exposed to blood-fed larvae, or exudate from molting ticks or untreated controls. Each mean represents 10 replicates with 10 nymphs per replicate.

[b] Significantly different from the untreated control, paired *t* test, $P < 0.001$.

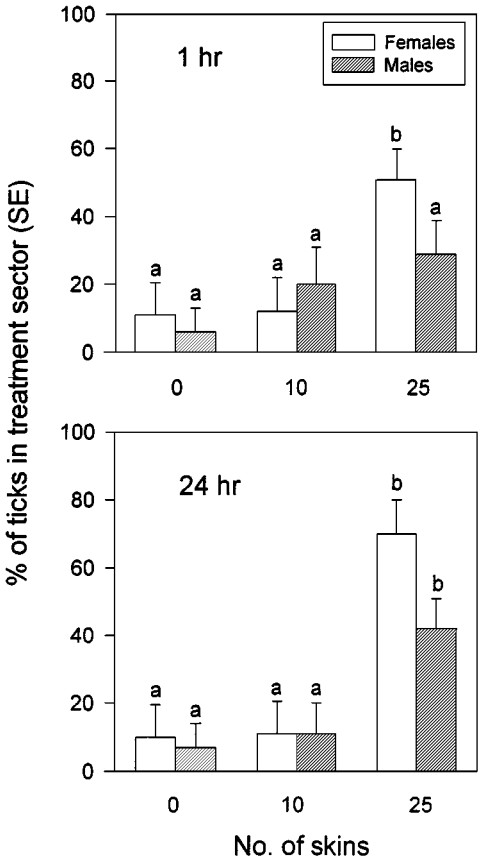


FIG. 1. Responses (mean ± SE) of adult *I. scapularis* to numbers of cast larval skins in Petri dish bioassays. Data are averages of 10 replications. Within each sex, bars marked by the same letter are not significantly different ($P > 0.05$).

Both male and female *I. scapularis* also responded to the presence of cast larval skins (Figure 1). In the presence of 10 larval skins, there was no difference after 1 hr in the number of male ($t = -0.6, df = 18, P = 0.27$) or female ($t = 0.24$, $df = 18$, $P = 0.40$) ticks in treatment sectors, nor was there a difference at 24 hr (male $t = 0.0$, $df = 18$, $P = 0.5$; female $t = 0.0$, $df = 18$, $P = 0.5$) compared to untreated controls. In the presence of 25 larval skins, there were more females in sectors with larval skins at both 1 hr ($t = -1.93$, $df = 18$, $P < 0.05$) and 24 hr ($t = -3.28$, $df = 18$, $P < 0.001$). There was no difference in numbers of males present in treatment sectors at 1 hr ($t = -0.89$, $df = 18$, $P = 0.19$) compared to untreated control sectors; however, there was a difference at 24 hr ($t = -1.4$, $df = 18$, $P < 0.05$).

TABLE 2. ASSEMBLY OF *I. scapularis* AFTER TWO-HOUR EXPOSURE TO CAST LARVAL SKINS, SALINE, OR METHANOL OR HEXANE EXTRACTS OF SKINS IN PETRI DISH BIOASSAYS[a]

Treatment	Ticks in treatment sector (zone 1) (%, mean ± SE)		
	Females	Males	Nymphs
Saline extract (95% NaCl)	64.3 (13.2)[b]	42.8 (13.7)[c]	45.0 (6.7)[b]
Methanol extract	10.0 (10.0)	10.0 (10.0)	10.0 (10.0)
Hexane extract	0.0 (0.0)	20.1 (13.1)	20.0 (13.3)
Cast skins	60.5 (13.3)[b]	40.5 (16.3)[c]	40.0 (7.3)[c]
Control	6.6 (6.6)	6.2 (6.2)	6.0 (3.1)

[a] Each treatment consisted of 20 skins or skin equivalents ($N = 10$).
[b] Significantly different from the untreated control, paired *t* test, $P < 0.001$.
[c] Significantly different from the untreated control, paired *t* test, $P < 0.05$.

Responses of ticks were similar to cast skins and to saline extracts of the skins (Table 2). More females were present in sectors with cast skins ($t = -3.85$, $df = 18$, $P < 0.001$) and the saline extract ($t = -3.02$, $df = 18$, $P < 0.001$) than the untreated controls. Similarly, responses of males and nymphs were greater to the cast skins ($t = -1.88$, $df = 18$, $P < 0.05$; $t = -4.29$, $df = 18$, $P < 0.001$, respectively) and the saline extract ($t = -2.37$, $df = 18$, $P < 0.05$; $t = -5.29$, $df = 18$, $P < 0.001$, respectively). Methanol and hexane extracts were not any more attractive than the controls ($P < 0.05$).

Assembly responses of nymphs were elicited by several of the purine standards tested (Table 3). More nymphs were present in sectors with guanine, uric acid, hypoxanthine, xanthine, inosine, and hematin compared to the solvent controls ($P < 0.05$). There was no significant response to adenine compared to the controls (Table 3). In general, responses increased slightly from 1 to 24 hr. After 24 hr, responses of nymphs to cast skins (40.0 ± 7.3%) were similar to responses to hematin ($t = 1.56$, $df = 18$, $P = 0.07$), xanthine ($t = 0.41$, $df = 18$, $P = 0.07$) and inosine ($t = 1.51$, $df = 18$, $P = 0.07$).

Similarly, responses of adults were also elicited by purine standards (Table 4). Uric acid, hypoxanthine, adenine, and hematin were also tested, but none of these elicited responses greater than the solvent controls ($P > 0.05$), and these data are not presented. More males and females were present in sectors with guanine, inosine, and xanthine compared to solvent controls (Table 4). Responses of females were similar to those obtained with cast skins (Table 2) and guanine ($t = 0.86$, $df = 18$, $P = 0.19$) and xanthine ($t = 0.86$, $df = 18$, $P = 0.18$); responses of males were similar between cast skins (Table 2) and guanine ($t = -0.86$, $df = 18$, $P = 0.19$), inosine ($t = 0.0$, $df = 18$, $P = 0.5$) and xanthine ($t = 0.0$, $df = 18$, $P = 0.5$).

TABLE 3. ASSEMBLY OF *I. scapularis* NYMPHS IN PETRI DISH BIOASSAYS IN RESPONSE TO PURINE COMPOUNDS (0.5 mg)[a]

	Ticks in the treatment sector (zone 1) (%, mean ± SE)							
Time (hr)	Control	Guanine	Uric acid	Hypoxanthine	Xanthine	Inosine	Adenine	Hematin
1	10.0 (4.4)	16.0 (5.0)	20.0 (5.1)	26.0 (5.1)[c]	22.0 (2.0)[c]	26.0 (4.0)[c]	6.5 (4.1)	24.0 (4.0)[b]
2	6.0 (3.0)	20.0 (4.8)[b]	30.0 (8.5)[c]	24.0 (5.6)[b]	28.0 (4.8)[c]	26.0 (2.4)[c]	16.0 (7.5)	24.0 (6.7)[c]
24	12.0 (4.4)	20.0 (5.0)	24.0 (6.0)	18.0 (6.6)	32.0 (3.7)[c]	36.0 (6.1)[c]	14.0 (4.1)	26.0 (5.6)[b]

[a] Each mean represents 10 replicates with 10 nymphs per replicate.
[b] Significantly different from the solvent control, paired *t* test, $P < 0.05$.
[c] Significantly different from the solvent control, $P < 0.001$.

TABLE 4. ASSEMBLY OF *I. scapularis* ADULTS TO PURINES IN PETRI DISH BIOASSAYS[a]

	Ticks in treatment sector (zone 1) (%, mean ± SE)							
	Females				Males			
Time (hr)	Control	Guanine	Inosine	Xanthine	Control	Guanine	Inosine	Xanthine
1	10.0 (10.0)	33.3 (12.6)	40.0 (13.1)[b]	33.3 (12.6)	6.0 (6.0)	33.3 (12.6)[b]	40.0 (13.1)[b]	26.6 (11.8)
2	6.0 (6.0)	33.3 (12.6)[b]	26.6 (11.8)	33.3 (12.6)[b]	6.0 (6.0)	40.0 (12.6)[b]	46.6 (13.3)[b]	33.3 (12.6)[b]
24	10.0 (10.0)	40.0 (16.4)[b]	20.0 (13.3)	40.0 (16.4)[b]	6.0 (6.0)	60.0 (16.4)[c]	40.0 (16.4)[b]	40.0 (16.0)[b]

[a]Each mean represents 15 replicates.
[b]Significantly different from the solvent control, paired *t* test, $P < 0.05$.
[c]Significantly different from the solvent control, paired *t* test, $P < 0.001$.

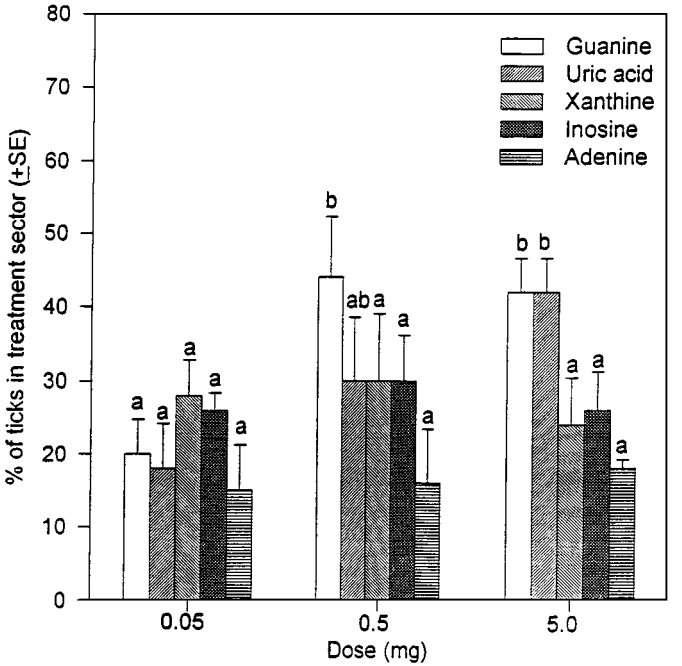


FIG. 2. Responses (mean ± SE) of *I. scapularis* nymphs to different concentrations of purines in a Petri dish bioassay. Data are averages of 10 means. Within each compound, bars marked by the same letter are not significantly different ($P > 0.05$).

Responses to several purine standards increased with dose when tested against nymphs (Figure 2). Differences in responses with increased dose were observed with guanine ($F = 5.51$, $df = 2, 29$, $P < 0.001$) and uric acid ($F = 3.43$, $df = 2, 29$, $P = 0.04$), but not with adenine ($F = 0.03$, $df = 2, 29$, $P = 0.96$), xanthine ($F = 0.20$, $df = 2, 29$, $P = 0.81$), or inosine ($F = 0.23$, $df = 2, 29$, $P = 0.29$).

Responses of nymphs to a range of concentrations of a mixture of guanine, xanthine, and adenine (25:1:1 ratio) differed (Figure 3) ($F = 2.55$, $df = 4, 74$, $P = 0.04$). Mixtures consisting of purines at 10.0, 1.0, and 0.1 mg elicited the highest responses (Figure 3). Responses of nymphs to cast skins were not different than responses to 10 mg and 1 mg (t test, $P > 0.05$) of purine mixtures, but were greater than other the other concentrations of purine mixtures tested (t test, $P < 0.05$).

DISCUSSION

This study provides clear evidence of an assembly pheromone in *Ixodes scapularis*. Similar to other reported assembly pheromones in ticks (summarized

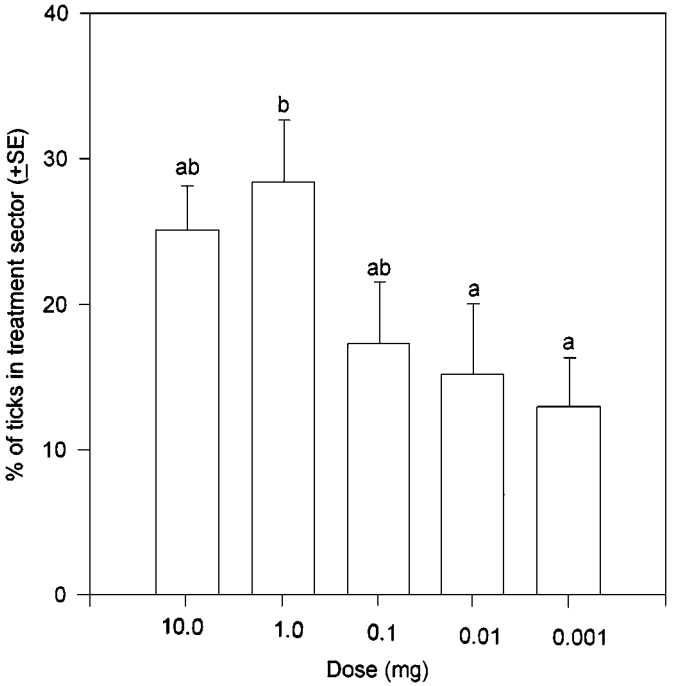


FIG. 3. Responses (mean ± SE) of *I. scapularis* nymphs to different concentrations of a mixture of guanine, xanthine, and adenine in a 25:1:1 ratio. Data are averages of 10 replicates in Petri dish bioassays. Bars marked by the same letter are not significantly different ($P > 0.05$).

in Sonenshine, 1991), arrestment responses were elicited by tick waste products (excreta and cast skins), responses could be elicited by saline but not organic solvent extracts, and responses were not stage-specific, with significant responses elicited from males, females, and nymphs. Additionally, equivalent assembly responses could be elicited in both nymphs and adults to several purine compounds previously identified as components of assembly pheromones in soft ticks (Dusbábek et al., 1991a).

Excretion of nitrogenous waste products by ticks generally occurs after blood-feeding and immediately after molting (thus contaminating the cast skins). Locations with an accumulation of excretory products are likely conducive to tick survival as these indicate successful completion of blood feeding and molting and have appropriate environmental conditions for tick survival (Otieno et al., 1985). Although there may be differences in sites of molting of different stages of *I. scapularis*, arrestment of tick movement in sites containing excretory products may result in assemblies of ticks of different stages since assembly pheromones are

nonspecific in activity. Although activity is nonspecific, there may be differences in quantities and mixtures of constituents produced between different stages. This study was conducted using cast skins from blood-fed larvae because of their use in previous studies and the ability to produce large numbers of them.

Nitrogenous waste products from ticks contain primarily guanine, other purines in lower concentrations and hematin (Hamdy, 1972, 1973). Dusbábek et al. (1991a) reported the relative abundance of guanine, xanthine, and hypoxanthine in excretory granules of five soft tick species. Several studies associated the observed assembly behaviors with excretory wastes of ticks (Leahy et al., 1973, 1975; George, 1981; Hájkova and Leahy, 1982; Otieno et al., 1985; Dobrotvorskii et al., 1991) as well as mites (Entrekin and Oliver, 1982; Arlian and Vyszenski-Moher, 1996). Aggregation in response to guanine was demonstrated in *Argas persicus* (Otieno et al., 1985), *Argas walkerae* (Neitz and Gothe, 1984), and *Argas persicus* (Hassanali et al., 1989). Response to guanine alone was reported by Gothe (1987) to be lower than to naturally occurring pheromone, supporting the idea that additional components may be present. Other compounds such as xanthine, hypoxanthine, and uric acid also elicited significant responses with response levels high in Otieno et al. (1985) (>75%). In addition to guanine, Dusbábek et al. (1991a) also reported significant assembly responses (>55%) of *A. persicus* males to xanthine, hypoxanthine, and inosine. Compounds tested that did not elicit assembly of ticks included adenine (Otieno et al., 1985; Dusbábek et al., 1991a), adenosine, guanosine, and uric acid (Dusbábek et al., 1991a). Several of these compounds, however, were reported to elicit assembly responses in *Sarcoptes* mites (adenine, uric acid) (Arlian and Vyszenski-Moher, 1996). Our data provide clear evidence that guanine, uric acid, hypoxanthine, xanthine, inosine, and hematin elicit assembly responses in *I. scapularis* in immatures and adults. However, whether any of these compounds occur in *I. scapularis* excreta and if so, their relative frequency, has yet to be determined.

Mixtures of purines appeared to elicit more assembly in the soft tick, *A. persicus*, compared to the purines alone (Dusbábek et al., 1991a). A mixture of guanine, adenine, and xanthine mixed in a ratio of 25:1:1 enhanced assembly of ticks equivalent to about 75% of the natural pheromone. Our data indicate that assembly of *I. scapularis* to a similar mixture of purines appears to be dose-dependent, and the highest responses to mixtures are equivalent to those of cast skins. The future identification of components and ratios in extracts may aid in further enhancement of assembly response in purine mixtures for *I. scapularis*.

Ixodes scapularis has emerged as a common suburban pest in the northeastern United States with the increased encroachment of communities into wooded areas (Lastavica et al., 1989; Carroll et al., 1992). Human risk for Lyme disease depends on the density of infected ticks as well as the degree of human–tick contact (Mather et al., 1989) with most risk associated with exposure to nymphs in summer (Spielman et al., 1985). Most tick control strategies are areawide and

do not specifically target ticks. The identification of assembly pheromones in *I. scapularis* can provide the basis for development of an alternative targeted control with the potential to reduce overall acaricide use and nontarget effects. The potential for such an approach was demonstrated by initial studies by Dusbábek et al. (1997), who reported the enhancement of efficacy of permethrin against immatures and adults of *A. persicus* when used in conjunction with guanine hydochloride.

Future research is needed to identify components in saline extracts containing the assembly pheromone and to determine a mixture of components equivalent in response to natural pheromone. Formulation of such components, in conjunction with an acaricide and perhaps also with volatile attractant compounds, may serve as the basis for development of a targeted control approach for *I. scapularis*.

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MATURE ATLANTIC SALMON (*Salmo salar* L.) MALE PARR ARE ATTRACTED TO OVULATED FEMALE URINE BUT NOT TO OVARIAN FLUID

K. HÅKAN OLSÉN,[1,4,*] ANNA-KARIN JOHANSSON,[1] RICKARD BJERSELIUS,[1] IAN MAYER,[2] and HANS KINDHAL[3]

[1]*Department of Environmental Toxicology, EBC*
Uppsala University
Norbyvägen 18A, SE-752 36 Uppsala, Sweden

[2]*Department of Zoology*
Stockhom University
SE-106 91 Stockholm, Sweden

[3]*Department of Obstetrics and Gynaecology*
Swedish University of Agricultural Sciences (SLU)
SE-750 07 Uppsala, Sweden

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Abstract—The behavioral responses to urine and ovarian fluids from conspecific and heterospecific ovulated females were studied in mature Atlantic salmon (*Salmo salar*) male parr in a two-choice fluviarium. The males reacted differently to the stimulants. They spent more time in water scented by urine from salmon or brown trout (*Salmo trutta L.*) females compared to the time in water with ovarian fluids from salmon females. Furthermore, the males were attracted to salmon female urine (different from an indifferent reaction). Males exposed to urine of either species had higher plasma concentrations of testosterone (T) compared to unexposed controls. Measurement of the concentrations of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its major metabolite 15-ketodihydroprostaglandin $F_{2\alpha}$ (15-ketodihydro-$PGF_{2\alpha}$) showed that the concentrations of the substances were higher in ovarian fluids of both species compared to those in urine. $PGF_{2\alpha}$ showed a greater difference between ovarian fluid and urine than its major metabolite. The results suggest that urine of both species, in contrast to ovarian fluid, contain substances that attract mature Atlantic salmon male parr and that the active substances are neither $PGF_{2\alpha}$ nor 15-keto-$PGF_{2\alpha}$.

*To whom correspondence should be addressed. E-mail: hakan.olsen@sh.se
[4]Present address: Department of Natural Sciences, Södertörns University College, Box 4104, SE-14104 Huddinge, Sweden.

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Key Words—Atlantic salmon, *Salmo salar*, brown trout, salmonids, sex odorants, precocious males, behavior, attraction, steroid hormones, pheromones.

INTRODUCTION

Previous studies have shown that mature salmonid females release odorants that affect the endocrine status (priming effect) and behavior (releasing effect) of mature conspecific males (for terminology see Liley, 1982). It has been previously shown that urine contains substances with priming effects in rainbow trout (*Oncorhynchus mykiss*) (e.g., Scott et al., 1994) and in Atlantic salmon (*Salmo salar*) (e.g., Waring et al., 1996). The males showed increased plasma sex steroid hormone levels, and this was frequently combined with enhanced volumes of milt. Other studies have given various results concerning the behavior responses of salmonid males to urine and ovarian fluids. Emanuel and Dodson (1979) induced upstream movements of rainbow trout males following exposure to ovarian fluids. Furthermore, Honda (1980, 1982) showed that odorants in the ovarian fluid of Pacific salmon stimulated different reproductive behaviors in males, and species specificity was suggested. Recent studies have shown that mature masu salmon males were attracted to water scented with urine from ovulated females, while ovarian fluids had no effect (Yambe et al., 1999). These contradicting results suggest that substances with effects on the male behavior are present both in the ovarian fluids and female urine, but the active source varied between the studies.

Recently we observed that mature male parr of Atlantic salmon and brown trout (*Salmo trutta*) were primed by odorants from ovulated females of either species (Olsén et al., 2000). The ovulated female stimulants, composed of odorants from a mixture of ovarian fluid and urine, were not species specific in these closely related species. Later we observed that both urine and ovarian fluid increased plasma sex steroid levels in mature male Atlantic salmon parr (Olsén et al., 2001). Both stimulants contained prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), that has been proposed to have a priming effect in mature Atlantic salmon parr (Moore and Waring, 1996). We have also shown that the plasma sex steroid hormones and volumes of strippable milt are increased in mature brown trout parr after exposure to F-prostaglandins (A. Moore, K. H. Olsén, and H. Kindahl, unpublished data).

In view of the above results, further studies are needed to determine whether both female urine and ovarian fluid are a source of releaser odorants in salmonids. In the present experiment, we studied the behavior and endocrine responses of mature Atlantic salmon parr exposed to low concentrations of urine or ovarian fluid from conspecific ovulated females, or from ovulated females of anadromous brown trout. We aimed at answering the questions: Are there any differences in response to urine and ovarian fluid and do salmon parr males respond differently to stimulants from conspecific and heterospecific females?

METHODS AND MATERIALS

Collection of Female Stimulants. Urine and ovarian fluids were collected by catheters from anesthetized (2-phenoxy ethanol, 0.05%) ovulated Atlantic salmon and brown trout females (Atlantic salmon: 80–107 cm T.L., 5.4–14.1 kg, $N = 8$; brown trout: 60–85 cm total length, 2.4–7.4 kg, $N = 17$) at the Älvkarleby hatchery on the River Dalälven. Both urine and ovarian fluids were taken from each female. The urine and ovarian fluids were collected in 20-ml polythene tubes on ice, and then stored at -80° C. Care was taken to prevent contamination between the two fluid types and involved using separate catheters. It was easy to fill the tubes with ovarian fluid, but the amounts of urine were often less than 10 ml. Prior to freezing, 0.5–1.5 ml of both fluids from seven females were decanted into microfuge tubes for analyses of $PGF_{2\alpha}$ and 15-Keto-dihydro-$PGF_{2\alpha}$ concentrations in individual fish. The remainder of the ovarian fluid and urine samples was pooled into an ovarian fluid mix and a urine mix, respectively, and frozen in aliquots suitable for the experiments.

Experimental Procedure. On October 13, 1999, one week before the start of the experiment, male parr with running milt were collected at Älvkarleby salmon hatchery and transported to Uppsala University and placed in 400-liter rearing tanks. Aerated tap water was continuously supplied and the photoperiod followed the natural cycle. Fish were fed Astra EWOS salmon feed. Individual fish (29.7–75.5 g, $N = 36$) were tested in a fluviarium at the Department of Environmental Toxicology (Höglund, 1961; Olsén, 1987; Bjerselius et al., 1995a,b) during the period October 20 to November 30, 1999. The fluviarium is an artificial stream with a laminar flow of aerated tap water. Water, with or without test stimulants, was supplied to opposite halves of the fluviarium and mixed into the main stream of aerated tap water flowing to the test area at a total rate of 18 liters/min (9 liters in each half) (Bjerselius et al., 1995a). The temperature in the fluviarium decreased during the experimental period from 10.7 to 9.2°C. Individual fish were placed in the test area within the fluviarium measuring, $46 \times 33 \times 11.5$ cm (L × W × D), formed by inserting two fine-mesh plastic screens. The test stimuli were released from 12-liter stainless steel containers by means of two peristaltic pumps, at the rate of 60 ml/min through two separate magnetic valves. Female stimulants were supplied from one container and only water from the other. In control tests, water was supplied from both. To ensure good mixing into the main stream, the supply of test stimuli from the stainless steel containers were mixed with a small supply of constantly flowing tap water (100 ml/min) through the magnetic valves. The supply in the fluviarium was also directed upstream. The two magnetic valves automatically switched the supply between the two halves of the test area at regular 90-min intervals. Then 4 ml of female stimulants (either salmon urine, trout urine, salmon ovarian fluid, or trout ovarian fluid) was supplied to 11 liters of water in the stainless steel container. Mixing in the main currents of the fluviarium was tested

using a green dye, and this showed that there was a sharp boundary line between the green-dyed and the nondyed sides in the test area.

A video camera was placed above the test area, and the position of the fish was recorded once every 2 sec by means of a video-computer image analysis system (EthoVision, Noldus Information Tech., Wageningen, The Netherlands) (Bjerselius et al., 1995a,b; Olsén and Winberg, 1996). Two 20-W halogen lamps equipped with a red glass filter (RG 780, Melles Giro) that allowed only transmission of wavelengths longer than 750 nm (beyond the visual range of salmonid fish) (Bone et al., 1995) were used as a light source for the camera. A full test started at 16:30 hr and ended at 07:30 hr the next morning. No stimuli were added during the first six 90-min periods (16:30–01:30 hr), but started at 01:30 hr and lasted until 07.30 hr. The water going through the magnetic valves shifted sides in the fluviarium every 90 min from the start to the end of the experiment (16:30–07:30 hr). It was only during the last four 90-min periods (01:30–07:30 hr) that the pumps added water with female stimulants or only water (control). The pumps ran every alternate 15-min period, starting at 01:45 hr, thereby giving three 15-min periods of stimulant release over each 90-min period. The intermittent pumping was intended to mimic the pulsatile nature of urine release female salmon (Curtis and Wood, 1991).

To reduce the risk of disturbance, the experiments were performed during the night. The stainless steel containers and the silicone medical grade tubes that supplied stimulants were rinsed with tap water, 95% ethanol, and tap water again after each test. The fluviarium was drained after each test and brushed with water and 95% ethanol.

For each fluviarium test, we used data collected for the 15-min periods between 01:30 and 07:30 hr, which corresponded to those periods of stimulant release, in addition to the corresponding periods during 19:30–01:30 hr. Based on the observations collected during these periods, a reaction value (R_v) was calculated for the periods 19:30–01:30 hr and 01:30–07:30 hr, respectively. R_v was calculated according to the following equation (Olsén, 1986):

$$R_v = [(N_L - N_0)/(N_L + N_0)] \times 100$$

where N_L and N_0 during 01:30–07:30 hr represent the number of observations in each side of the test area when supplied with water containing female stimulants, or water only, respectively. During the period 19:30–01:30 hr, only water without help from the pumps passed through the magnetic valves. The arithmetic means of reaction values from identical tests are designated MR_v. A positive R_v during 01:30–07:00 hr means that the fish spent more time in the half of the fluviarium with the stimulant added than in the side with tap water added from the second stainless steel tank. A positive R_v during 19:30–01:30 hr means that the fish were more frequently observed in the half that was supplied through the magnetic valve that later (01:30–07:00 hr) was supplied with stimulant.

Blood Sampling. After each fluviarium test, blood was sampled from all fish as follows. For each sample, the male parr was first anesthetized (2-phenoxyethanol, 0.05%), weighed, and the volume of milt determined by collecting it in preweighed glass tubes following gentle abdominal pressure (Olsén and Liley, 1993). Blood was subsequently collected by severing the caudal peduncle and collecting the blood in heparinized tubes. The fish were humanely killed after blood sampling. Following centrifugation, the plasma was collected and stored at -80°C for future sex steroid determination. The testes were weighed for the determination of gonadosomatic index [GSI (gonad weight/body weight) $\times$ 100].

Hormone Measurements. Plasma levels of 11-ketotestosterone (11-KT), testosterone (T), and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) were measured by specific radioimmunoassay (RIA) according to the method described by Mayer et al. (1990).

The metabolite of prostaglandin $F_{2\alpha}$, 15-ketodihydro-$PGF_{2\alpha}$, was analyzed in unextracted media by RIA (Kindahl et al., 1976; Granström and Kindahl, 1982). The antiserum cross-reacted 16% against 15-keto-$PGF_{2\alpha}$, 4% against 13,14-dihydro-$PGF_{2\alpha}$, 0.4% against $PGF_{2\alpha}$, and 1.7% with 15-ketodihydro-PGE_2, a major metabolite of PGE_2. For other prostaglandins tested, the cross-reaction was $<$0.1%. The samples were diluted in a 0.25% bovine γ-globulin buffer. In most cases, 50 μl of media was diluted with 450 μl of the buffer. The practical detection limit of the assay was 300 pmol/liter. If necessary, the samples were diluted to allow accurate readings on the standard curve. The interassay coefficient of variation was 14%. Samples were reanalyzed if the coefficient of variation exceeded 10% for the duplicate determinations. The intraassay coefficient of variation was evaluated by analyses of 10 different pooled plasma samples in five separate radioimmunoassay tubes and was between 3.4% and 7.6% at different ranges of the standard curve.

The primary prostaglandin $F_{2\alpha}$ was analyzed in the media by a modified method according to Lindgren et al. (1974). The samples were diluted in the same way as for the analysis of 15-ketodihydro-$PGF_{2\alpha}$ as described above. The antiserum against $PGF_{2\alpha}$ cross-reacted 75% against $PGF_{1\alpha}$, 2% against $PGF_{2\beta}$, 1.5% against $PGF_{1\beta}$, and $<$0.1% for PGE_2, PGE_1, PGD_2, TXB_2, and 15-ketodihydro-$PGF_{2\alpha}$. The practical limit of detection for $PGF_{2\alpha}$ was 0.3 nmol/liter. The interassay coefficient of variation was 15% and the intraassay coefficients of variation varied between 7.3% and 12.5% at different ranges of the standard curve.

Statistical Analyses. Reaction values, plasma hormone concentrations, milt volumes, and GSIs were compared among treatments with a one-way ANOVA. In cases of significance, the ANOVA was followed by post tests. The behavior analyses were followed by Newman-Keuls multiple comparison test. Dunnett's test was used when comparing the plasma steroid hormone levels in males to different exposures with the concentrations in unexposed control males. The calculations were performed with GraphPad Prism (Motulsky, 1995, 1999).

Student's *t* test was used when the MR_{vs} from fluviarium tests were compared with the theoretically indifferent reaction, i.e., when $MR_v = 0$. MR_v is zero when the number of observations in each water quality is the same.

RESULTS

Behavior. ANOVA showed that there were significant differences in behavior among the different treatments ($F_{4,31} = 3.751$, $P = 0.0133$). Newman-Keuls test revealed differences between the responses to salmon urine and ovarian fluid from salmon ($q = 3.986$, $P < 0.05$) and between trout urine and salmon ovarian fluids ($q = 4.654$, $P < 0.05$) (Figure 1).

Males were attracted to female salmon urine (MR_v was different from an indifferent response when $MR_v = 0$) (Figure 1). No behavioral responses were observed with the other stimulants. Neither was there any preference for either of the two channels entering through the magnet valves to either side of the fluviarium before the supply of stimulants (within 90-min periods 3–6).

Blood Plasma Levels of Sex Steroids. A one-way ANOVA showed that there were significant differences in plasma T levels among the males undergoing different treatments ($F_{4,31} = 3.186$, $P = 0.0265$). The males that had been in contact with salmon or trout female urine had higher plasma concentrations of T than control males (Figure 2). Treated males were only compared with control males (Dunnett's test). No differences in plasma concentrations of 17,20β-P or 11-KT were observed among males given different treatments (17,20β-P: $F_{4,31} = 1.268$, $P > 0.05$; 11-KT: $F_{4,31} = 1.295$, $P > 0.05$).

Milt Volumes and GSI. No differences in volumes of strippable milt were observed between the controls and the fish exposed to the different female odors (milligrams of milt per gram of fish; mean $\pm$ SD; control: 28.3 ± 14.8, $N = 8$; salmon urine: 34.2 ± 12.9, $N = 7$; salmon ovarian fluid: 32.9 ± 9.4, $N = 7$; trout urine: 32.7 ± 16.8, $N = 7$; trout ovarian fluid: 25.4 ± 7.4, $N = 7$; $F_{4,31} = 0.60$, $P > 0.05$). There were also no differences in GSI (control: 9.1 ± 1.6, $N = 8$; salmon urine: 9.4 ± 1.4, $N = 7$; salmon ovarian fluid: 9.8 ± 0.8, $N = 7$; trout urine: 10.1 ± 1.8, $N = 7$; trout ovarian fluid: 9.0 ± 0.7, $N = 7$; $F_{4,31} = 0.94$, $P > 0.05$).

Prostaglandin Concentrations in Urine and Ovarian Fluid. Seven females of each species were analyzed. The median concentration of $PGF_{2\alpha}$ in the urine of salmon was 11.4 nmol/liter (range 5.4–16.1 nmol/liter). All samples of ovarian fluid from salmon contained $PGF_{2\alpha}$, with concentrations over 200 nmol/liter. In trout females, the median concentration of $PGF_{2\alpha}$ in urine was 0.4 nmol/liter (range 0.3–8.4 nmol/liter). In ovarian fluid, the concentrations of $PGF_{2\alpha}$ were greater than 200 nmol/liter in all but two samples, which had levels of 117 and 153 nmol/liter. The median concentration of 15-ketodihydro-$PGF_{2\alpha}$ in salmon

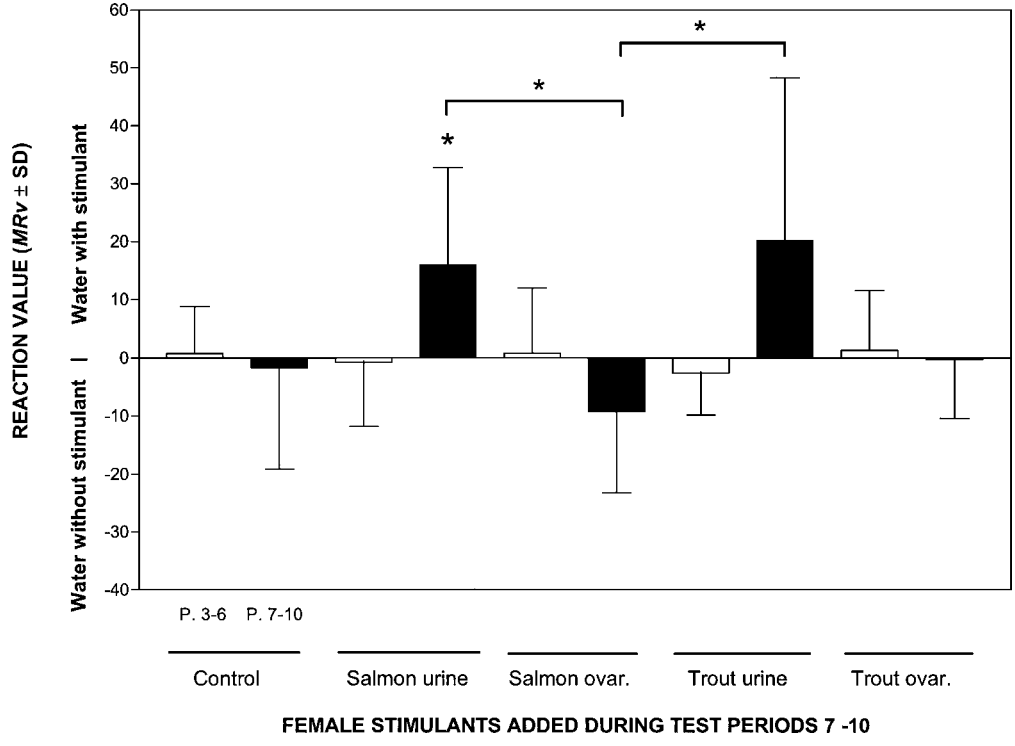


FIG. 1. Reaction value ($MR_v \pm$ SD) of mature Atlantic salmon male parr exposed to water scented by urine or ovarian fluid from conspecific or brown trout females. Only tap water was used in control tests. Positive MR_v means that the test fish is attracted to the stimulant. A significant difference between treatments is denoted by asterisk. An asterisk above the bar states that the MR_v was significantly different from an indifferent reaction when $MR_v = 0$. The open and solid columns represent the reaction values during the test periods 3–6 and 7–10, respectively. Seven fish were used for each treatment, except for controls which used eight. $^*P < 0.05$.

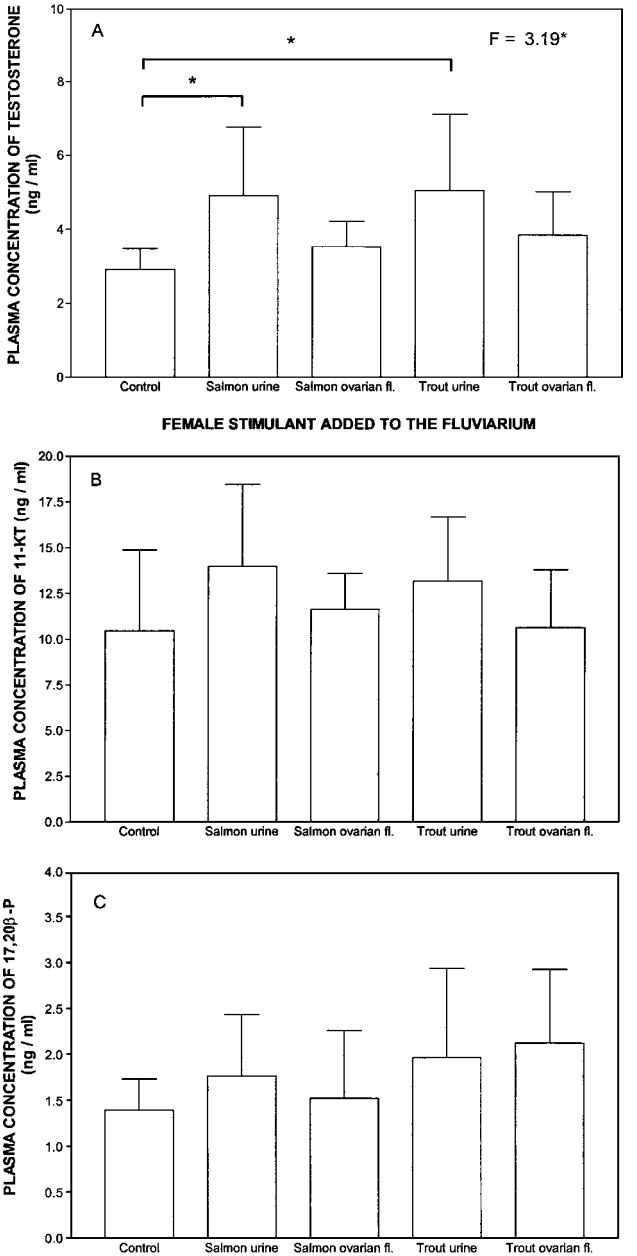


FIG. 2. Plasma steroid hormone levels are shown in mature Atlantic salmon male parr after exposure to urine or ovarian fluid from conspecific or heterospecific ovulated females or only to tap water (control) in fluviarium tests. The hormone levels after each exposure were compared to the control. The same number of fish used as in Figure 1. Values are mean $\pm$ SD, $^*P < 0.05$.

urine and ovarian fluid was 2.0 nmol/liter (range 1.3–5.5 nmol/liter) and 19.5 nmol/liter (range 15.6–48.0 nmol/liter), respectively. In trout females, the median concentration of the metabolite was 0.7 nmol/liter in urine (range 0.2–1.1 nmol/liter) and 2.6 nmol/liter in ovarian fluid (range 1.0–5.7 nmol/liter).

DISCUSSION

The present study shows that urine from ovulated females contains odorants that attract mature Atlantic salmon male parr. The ovarian fluids of both species were not attractive and, if anything, were somewhat repellent. The males that were exposed to urine of both species had higher plasma concentrations of T compared to the controls. These results are in agreement with those of Yambe et al. (1999) on masu salmon. They showed that attractants are present in the urine of ovulated females, but not in ovarian fluid. Furthermore, the male masu salmon were not attracted to the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which also agrees with our results with salmon parr (Olsén et al., 2001). In contrast, $PGF_{2\alpha}$ has been shown to be a female attractant in Arctic charr (*Salvelinus alpinus*) (Sveinsson and Hara, 1995). Yambe and Yamazaki (2000) also found that juvenile masu males treated with methyl-testosterone were attracted to ovulated female urine. In the present study, the higher plasma concentrations of T were probably caused by the urine exposure. Previous studies have shown that exposure to female urine (Waring et al., 1996), or a mix of urine and ovarian fluid (Olsén et al., 2000), increases the plasma sex steroid levels and the volumes of strippable milt in mature Atlantic salmon male parr. Results have suggested that $PGF_{2\alpha}$ is the priming chemical in Atlantic salmon or is an important part of it (Moore and Waring, 1996; Olsén et al., 2001). In addition to urine, ovarian fluid also has a priming effect on salmon male parr, and both stimulants contain $PGF_{2\alpha}$ (Olsén et al., 2001). The dilution steps of urine and ovarian fluid in the present study should have resulted in low concentrations of $PGF_{2\alpha}$ in the fluviarium. We observed that the concentration of $PGF_{2\alpha}$ was at least 20 times higher in ovarian fluid than in urine of Atlantic salmon females. In brown trout, the concentration was 500 times higher in the ovarian fluids. In an earlier study, we observed that the concentration of $PGF_{2\alpha}$ in ovarian fluid of Atlantic salmon was about a 1000 times higher than in urine (500 nmol/liter vs 0.4 nmol/liter) (Olsén et al., 2001). Based on these measurements, the concentrations of $PGF_{2\alpha}$ should have been about 1 pM and 0.001–0.1 pM in the test area with ovarian fluid and urine, respectively. These concentrations are probably too low to be detected by males (Moore and Waring 1996; K. H. Olsén, P. W. Sorensen, and C. Murphy, unpublished data). The results suggest that $PGF_{2\alpha}$ is not the female urine attractant for Atlantic salmon male parr. The active attractants in our study appear to be present in significant concentrations only in the urine. The results also suggest that $PGF_{2\alpha}$ is not the priming chemical

that gave increased plasma testosterone concentrations after urine exposure but not after ovarian fluid.

To date, very few chemicals released by female fish that have an effect on the behavior of conspecific males have been identified. F-type prostaglandins induced reproductive behavior in goldfish (*Carassius auratus*) (e.g., Sorensen et al., 1989) and cobitid loach (*Misgurnus anguillicaudatus*) (Kitamura et al., 1994), and steroid glucuronides are active as releaser pheromones in the African catfish *Clarias gariepinus* (Resink et al., 1989). In masu salmon, the female attractant present in urine is not a free steroid or $PGF_{2\alpha}$ (Yambe et al., 1999).

At present, we do not know the chemical identity of the active attractants in Atlantic salmon and brown trout female urine. One candidate might be T, which is detected by the olfactory epithelium in mature male Atlantic salmon parr at concentrations as low as 10^{-14} M (Moore and Scott, 1991) and has been shown to act as an attractant to the males (Moore, 1991). Moore and Scott (1992) also referred to unpublished data that female urine induced positive rheotactic behavior in precocious Atlantic salmon male parr, similar to that induced by T. The next step in our study will be to analyze the release of different conjugated steroids in the urine of females and test their potency as attractants in mature male parr. Salmonids release both free steroid hormones and their metabolites in the urine (c.f. Scott and Liley, 1994).

In summary, the present study shows that urine from ovulated female salmon and possibly brown trout, contains a chemicals that attract mature male salmon parr and stimulates increased plasma testosterone levels. Ovarian fluid appears to not have such effects. Furthermore, we do not have any evidence that $PGF_{2\alpha}$ is the active substance.

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GAS-CHROMATOGRAPHIC ANALYSES OF THE SUBCAUDAL GLAND SECRETION OF THE EUROPEAN BADGER (*Meles meles*) PART I: CHEMICAL DIFFERENCES RELATED TO INDIVIDUAL PARAMETERS

C. D. BUESCHING,[1,*] J. S. WATERHOUSE,[2] and D. W. MACDONALD[1]

[1]*Wildlife Conservation Research Unit*
Department of Zoology
University of Oxford
South Parks Road, Oxford, OX1 3PS, UK

[2]*Environmental Sciences Research Centre*
Anglia Polytechnic University
East Road, Cambridge, CB1 1PT, UK

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Abstract—In many species, chemical signals are important in the context of intraspecific communication. European badgers (*Meles meles*) use the secretion of their subcaudal gland to mark both the environment and conspecifics. In this paper, we investigated the chemical composition of subcaudal gland secretions and how it varies among individuals according to group membership, season, sex, age, body condition, and reproductive status. We analyzed 66 samples by gas chromatography using a mass-spectrometer as detector. We found 110 different components, of which 21 were present in every profile. The chemical composition of the secretions proved to be highly individual-specific, but no single peak was found to be typical for one of the investigated categories (season, sex, etc.). Analyses of the relative area (% area) that every peak contributed to the overall profile area showed that, in general, group members have more similar profiles than do badgers from different groups. Composition varied over seasons and between sexes and was influenced by age, body condition, and reproductive status. We, therefore, conclude that the secretion not only encodes group membership and individuality as previously assumed, but might also convey information about fitness-related parameters.

Key Words—Badger, body odor, carboxylic acid, chemical communication, individuality, Mustelidae, chemical signal, scent-marking, semiochemistry.

*To whom correspondence should be addressed.

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INTRODUCTION

The subcaudal gland of the European badger (*Meles meles*) is a large skin gland, that is unique to this species (Brown and Macdonald, 1985). Sebaceous (holocrine) and sudiferous (apocrine) secretory tissues empty their pastelike secretion into a common lumen, the subcaudal pouch, which opens into the environment through a 20- to 80-mm-wide horizontal slit situated between the tail and the anus. In this paper, we report on the kind of information that is likely to be contained in the subcaudal secretion, and how chemical composition varies according to individuality, group membership, season, sex, and fitness-related parameters ("fitness"), such as age, body condition, and reproductive status.

Badgers use subcaudal gland secretions to scent-mark conspecifics (allomarking; Buesching et al., unpublished data), bedding material and other objects, the ground around their sett, paths, and latrines (object-marking; Buesching, 2000). A number of functions have been suggested for subcaudal scent-marking, including territorial signaling (Kruuk, 1978), group membership (Gorman et al., 1984), individual recognition (Kruuk et al., 1984), and fitness advertisement (Buesching and Macdonald, 2001). However, the chemical information encoded in subcaudal secretions is not yet fully understood. The only analytical study based on gas chromatography (Gorman et al., 1984) confirmed interindividual differences in the chemical profiles and showed the existence of group-specific characteristics in the scent profiles, thus suggesting that badgers can obtain information about individuality and the group membership of conspecifics from subcaudal scent-marks. Yet, despite significant sex- and fitness-related differences in the secretion color throughout the year (Buesching et al., 2002a), the above-mentioned study failed to show any sex- or fitness-related differences in the chemical profiles. One possible explanation for this result could be that Gorman et al. (1984) compared samples collected at different times of the year. Hence, possible sex- and fitness-related characteristics might have been obscured by overlying seasonal variation. The aim of the present study is, therefore, to compare samples collected within the same calendar month and to investigate whether differences in the chemical profiles can be related to season, sex, and "fitness."

METHODS AND MATERIALS

Study Animals and Collection of Samples. A total of 66 samples were collected from 35 females and 31 males during four trapping events between January and November 1998 from a badger population in Wytham Woods, Oxfordshire, England (Ordnance survey reference: SP462080; for detailed description of the study site see Kruuk, 1989). Cubs (i.e., individuals younger than 1 year) were not included in this study. Badgers were trapped following the methodology described

by MacDonald and Newman (2001). At first capture (usually as cubs), all individuals were tattooed with an individual number on the left inguinal region. Thus, after anesthesia with 0.2 ml ketamine hydrochloride (Vetalar, Pharmacia and Upjohns Ltd., Crawley, E. Sussex, UK) per kilogram body weight (Hunt, 1976), all badgers could be identified individually, sexed, and aged [category 1, cubs younger than 1 year (*excluded from this study*); 2, yearlings 1–2 years old; 3, adults 2–5 years old; 4, adults older than 5 years], and their body condition determined using a subcutaneous fat score originally developed for sheep (with 1 being emaciated and 5 being in very good condition; modified from Speedy, 1980). In males, reproductive status was determined by the degree to which the testes were descended from the body cavity into the scrotum (Woodroffe and MacDonald, 1995) and classified as 1, fully descended testes: in breeding condition; 2, intermediate: reproductive activity could not be determined unambiguously; and 3, completely retracted testes: not in breeding condition. In females pregnancy was determined by ultrasound scans (Kontron: external 5.0 MHz sector probe) in winter, and in spring, teats were measured for length and diameter to determine (recent) lactation (Cheeseman et al., 1987; Woodroffe, 1993), if no milk could be expressed.

Secretion was scooped out of the subcaudal pouch using a rounded stainless steel spatula. All samples were frozen immediately and stored in glass vials at -70°C for a maximum of one year until further analysis.

Gas-Chromatographic Analyses. Samples were analyzed using a Hewlett-Packard 5890A gas chromatograph (GC) with a polydimethylsiloxane column (25 m $\times$ 0.32 mm ID, 0.5-μm film thickness) and a Hewlett-Packard mass spectrometer (MS; HP5970 Series Mass Selective Detector) as detector.

After solvent extraction with hexane (approx. 1 g secretion in 0.2 ml hexane), the extract was left to settle for approx. 3 min. The supernatant was transferred into a second glass vial and mixed with one spatula tip of dried magnesium sulfate to trap the water content. The extract was filtered through a syringe filter (0.2-μm pore size) and injected directly into the column (1 μl; start temperature 40°C; temperature rise 8°C/min; end-temperature 200°C for 10 min with a solvent delay of 2 min). After each sample, the GC was heated to 240°C for 10 min, and after every four samples, a blank was run to ensure that no contamination was left in the column. Results were saved automatically using the Hewlett-Packard software G1034C version C.03.00, and, where possible, chemical compounds were identified by their retention times and mass spectra.

Statistical Analyses of GC Profiles. To compare different profiles, peaks were matched by their retention times and mass spectra. Injection of a mixture of nine standard acids (tetranoic, hexanoic, linoleic, octanoic, nonanoic, decanoic, undecanoic, dodecanoic, and hexadecanoic acid), and linoleic ethyl ester were used to validate retention times. Compounds were identified primarily from their mass spectra, and without confirmation these identities remain tentative. For some of the components (see Table 1), it was possible to confirm identities by comparison of

TABLE 1. CHEMICAL COMPONENTS PRESENT IN EVERY PROFILE[a]

Peak	Confirmed (coinjection)	Chemical component	% area (mean ± SD)
5		methylundecanoic acid? (branched C_{12} acid)	1.965 ± 1.55
11		***???	0.465 ± 0.40
13		***???	1.400 ± 1.01
21	yes	dodecanoic acid	0.637 ± 1.01
24		3(?)-methyldodecanoic acid	2.745 ± 1.98
29		???	1.333 ± 0.74
43	yes	tetradecanoic acid	2.523 ± 1.30
47		???	0.745 ± 0.53
48		3(?)-methyltetradecanoic acid	3.626 ± 2.72
50		14(?)-methylpentadecanoic acid	1.975 ± 1.47
56		(branched C_{16} acid)	10.083 ± 3.95
68	yes	hexadecanoic acid	30.576 ± 8.97
75		***???	0.903 ± 0.81
77			
78			
79			
77–79		14(?)-methyldecanoic acid + ???	2.257 ± 1.51
83	yes	heptadecanoic acid + branched C_{18} acid	4.027 ± 2.32
89	yes	linoleic acid	7.297 ± 3.71
90		mono-unsaturated C_{16} acid (?)	4.252 ± 2.66
91		mono-unsaturated C_{16} acid (?)	2.416 ± 1.66
95	yes	octodecanoic acid	8.397 ± 4.47

[a](*** = components with significant MS peak at 114; ??? as yet unidentified compounds).

GC times with those of authentic compounds. Some peaks (11, 13, 47, 75) could not be identified from mass spectra, and further analytical work is being carried out. The contribution of each peak to the overall area of the whole profile was calculated in percent (% area) and used for analyses. Peaks contributing less than 0.1% to the overall profile area were excluded from all analyses. If a certain peak could not be detected in a given profile, its % area was set as 0.001% to enable further statistical analyses, which would not accept values of zero. Independent samples were used for all analyses (i.e., no individual contributed more than one sample to the data set). Statistical analyses were carried out using Statistica for Windows (Release 4.5, StatSoft Inc. 1993).

RESULTS

In total we found 110 different components, of which 21 were present in all samples (Table 1); 16 components occurred only once in the data set, and 9 were

present in only two individuals. No pattern characterizing any of the categories investigated (sex, season, age, body condition, reproductive status) could be found in the presence/absence of any of the 89 peaks, which was not present in all individuals. Therefore, all analyses, except those concerning group similarities and average peak numbers, were restricted to the 21 shared peaks, which were present in every sample (the majority of which are carboxylic acids) and are listed in Table 1. However, due to overlap between peaks 77, 78, and 79, the respective % area of these three peaks—although present in every profile—could not always be clearly defined. Therefore, their values were combined in all further analyses, except those concerning average numbers of peaks per profile. Peak 83 proved to comprise two different components (heptadecanoic acid and a branched C_{18} acid), that were too similar in their retention times to be separated in this study and are, thus, combined in all analyses.

Compounds were identified primarily from mass spectra, and without confirmation these identities remain tentative. For some of the components (see Table 1), it was possible to confirm identities by comparison of GC retention times with those of authentic compounds. Some peaks (11, 13, 47, 75) could not be identified from their mass spectra. Although several of these components (marked with ??? in Table 1) show a significant peak at 114, they are not carboxylic acids, and their chemical structures are as yet unknown. Although the % area of several of the 21 shared peaks proved to be significantly correlated with one another, no correlation was significant for each of the investigated sex/season subsamples. Therefore, we separately performed all subsequent analyses for each peak.

Individuality. Chemical composition of subcaudal gland secretions proved to be highly individual-specific. The chemical profile of each badger consisted of a unique combination of 23–58 different peaks (average over all samples 33.4 ± 8.05, $N = 66$), and relative % area of all peaks varied highly between individuals (Table 1; Figure 1).

Group Membership. A cluster analysis (unweighted pair-group average) including all peaks (presence/absence and % area) occurring in more than one sample was performed on the secretions of 46 badgers from seven well-defined social groups (Figure 2). Results indicated greater similarities between the profiles of badgers from the same social group than from different groups, which is reflected in the tendency of group members to cluster closer together.

Due to the high number of variables and the influence of other parameters on the composition of subcaudal gland secretions (see below), this analysis can only reveal broad similarities. To investigate further group-related differences in the chemistry of subcaudal gland secretions we performed an ANOVA on the same data set, that showed that four peaks varied between groups (P5: $F\,(df\ 1,2)\ 7,43 = 3.240$, $P < 0.01$; P48: $F\,(1,2)\ 7,43 = 3.892$, $P < 0.005$; P50: $F\,(1,2)\ 7,43 = 2.321$; $P < 0.05$; P90: $F\,(1,2)\ 7,43 = 3.600$, $P < 0.005$).

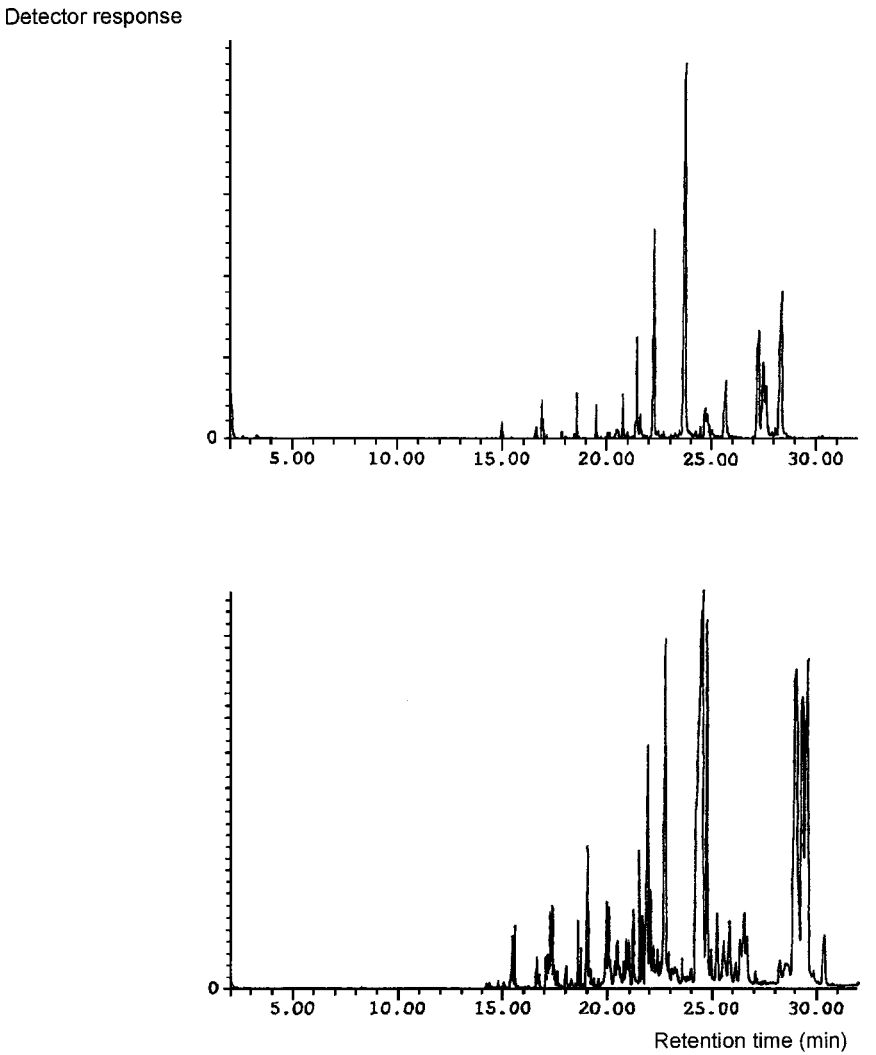


FIG. 1. Gas chromatography profiles of two female badgers of similar age illustrating interindividual variation (capture date: June 20, 1998).

Seasonal and Sexual Variation. The physiology of badgers is strongly influenced by season. The main mating season is between January and March during a post-partum estrous, and cubs are weaned around mid-April (Neal, 1990). A massive weight gain in autumn is followed by gradual decrease in body condition over winter, and badgers usually reach their minimal body weight around August

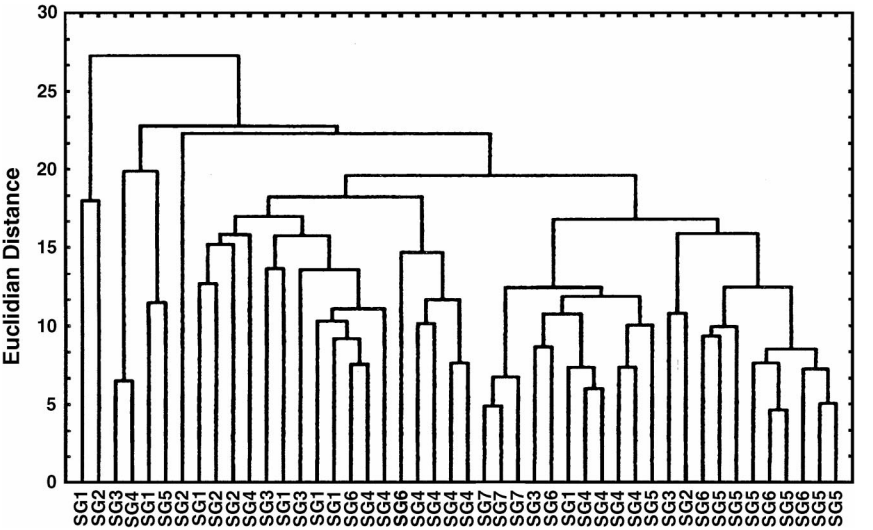


FIG. 2. Dendrogram of cluster analysis for 46 badgers from seven different social groups (SG = social group).

(Kruuk and Parish, 1983). It was noted that extracts derived from summer and autumn samples were far richer in residues (presumably proteins; Gorman et al., 1984), but at the same time much less concentrated in their chemical composition than those derived from winter and spring samples, this resulted in the need to condense by evaporation summer and autumn extracts to approximately 20% of their original volume. However, the experimental design of this study did not allow detailed quantitative analysis of the scent concentration in the samples.

To evaluate the effect of season on the composition of subcaudal gland secretions, we performed a two-way MANOVA with season and sex as factors, and corrected for age and body condition (= covariates). We employed contrast analyses to specify seasonal effects on male and female *secretions* and to test for sexual differences in the chemical composition over seasons (Table 2).

Chemical composition varied over season and between sexes (Table 2). Generally, profiles of female badgers comprised more peaks than did those of males ($x_{\text{females}} = 36.1, x_{\text{males}} = 30.7; U = 390.5, Z_{\text{adj}} = -1.956, P = 0.05$; Mann-Whitney-U test). However, while in males no seasonal differences were found in the number of peaks per profile, females had less complex profiles in spring than in winter ($U = 18$, $Z_{\text{adj}} = -2.583$, $P = 0.01$; Mann-Whitney-U test) and summer ($U = 15$, $Z_{\text{adj}} = -2.136$, $P < 0.05$; Mann-Whitney-U test).

In both sexes, the % area of peak 24 was lowest in winter and highest in autumn, and throughout most of the year it contributed less to the overall profile

TABLE 2. SIGNIFICANT RESULTS OF MANOVA WITH SEX AND SEASON AS VARIABLES[a]

Peak	Seasonal effect in		Effect of sex over season $F\,(df = 1,55)$
	Males $F\,(df = 1,55)$	Females $F\,(df = 1,55)$	
P24	12.656***	24.376***	11.293**
P43	9.733**		14.837***
P48	17.370***	29.305***	8.981**
P50	8.842**	16.713***	7.349**
P56	9.533**	8.061**	
P68	35.000***	23.677***	8.371**
P77–79	6.340*	19.230***	21.508***
P83			5.868*
P89	14.064***	15.112***	
P90	5.857*	5.729*	
P91	4.606*		
P95	4.874*		4.470*

[a] ($F_{1,2} = 3.58$) Significance levels $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

area in males than it did in females. However, this difference was not significant in autumn. The % area of peak 43 showed only little seasonal variation, but was consistently higher in males than in females. This difference was most pronounced in winter ($F = 13.67$, $P < 0.001$). Whereas in females, the relative % area of peak 48 and peak 50 increased consistently throughout the year, in males they stayed on a low level from winter to summer and increased sharply towards autumn. Both peaks were always lower in males than in females. The relative % area of peak 68 showed seasonal variation only in females, but was consistently high in males. During winter, summer, and autumn, its contribution to the overall profile was lower in females than in males. However, in females its % area showed a marked increase in spring, thus reaching the same levels as in males. Strongest sexual differences were found in peak 77–79 (Figure 3). Whereas in females, its % area was low during winter and then climbed to a higher level from spring to autumn, in males it was highest in autumn and winter and reached a relative minimum in spring. Except during winter, these peaks contributed less to the profiles in males than in females. In females, peak 83 peaked in summer, but showed no variation among any other seasons. In males, its % area was lower in spring than in winter, and lower than in summer and autumn. In males, this pattern was also repeated in peak 95, whereas in females it was higher in winter than during other seasons.

Effects of Individual-Specific Parameters. Age showed a strong influence on the chemical composition of subcaudal gland secretions of badgers. The % area of peak 83 was positively correlated with age in both sexes (females: $N = 44$, $R = 0.443$, $P < 0.005$; males: $N = 43$, $R = 0.388$, $P = 0.01$; Spearman rank order correlation), with older (>5 years) individuals having larger % area

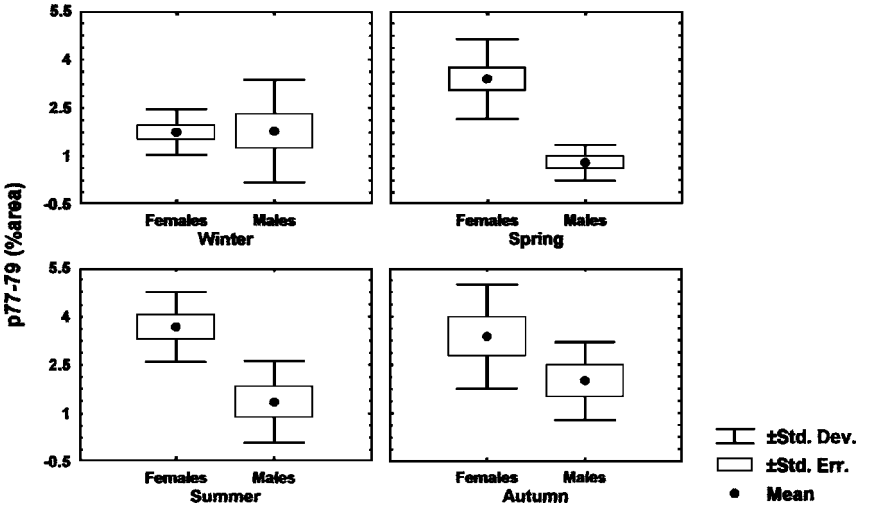


FIG. 3. Sexual differences over season in the % area of peaks 77–79.

than yearlings (females: $U = 15$, $Z_{adj} = -1.967$; $P < 0.05$; males: $U = 7$, $Z_{adj} = -2.061$, $P < 0.05$; Mann-Whitney U test; Figure 4). No relationship between age and average number of peaks per profile became apparent (females: $U = 17.5$, $Z_{adj} = -0.795$, $P = 0.427$; males: $U = 11$, $Z_{adj} = -0.851$, $P = 0.395$; Mann-Whitney U test).

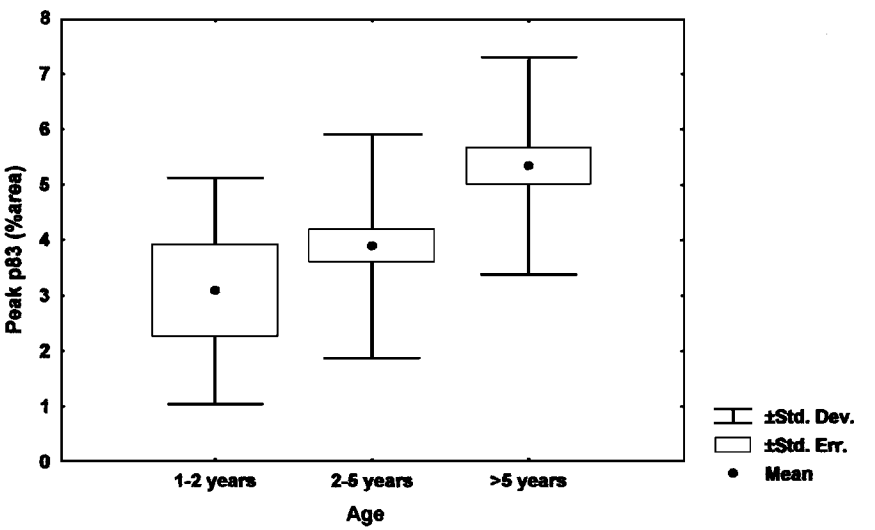


FIG. 4. Correlation between % area of peak 83 and age.

Body Condition was correlated with relative peak areas only in winter and summer. In females, peak 5 ($R = 0.844$, $P < 0.005$) and peak 50 ($R = 0.683$, $P < 0.05$) were positively correlated with body condition in winter, and peak 47 ($R = 0.845$, $P < 0.05$) and peak 75 ($R = 0.845$, $P < 0.05$) in summer. In males, peak 75 ($R = 0.648$, $P < 0.05$) and peak 89 ($R = 0.752$, $P < 0.05$) were correlated with body condition in winter, and peak 56 ($R = 0.850$, $P < 0.05$) and peak 89 ($R = -0.756$, $P < 0.05$; Spearman Rank correlation) in summer. While in females the number of peaks was not related to body condition, males in better body condition had fewer peaks per profile in winter ($N = 9$, $R = -0.684$, $P < 0.05$) and in spring ($N = 9$, $R = -0.872$, $P < 0.005$; Spearman rank correlation) than had thinner individuals, coinciding with the reproductive period.

Reproductive status had a significant influence on the chemical composition of subcaudal secretions in both sexes. In pregnant females, peak 83 contributed less to the overall profile area than it did in nonpregnant individuals ($U = 8$; $Z_{adj} = -2.066$, $P < 0.05$; Mann-Whitney U test; Figure 4), and it was smaller in lactating than in nonlactating females ($U = 9$, $Z_{adj} = -2.382$, $P < 0.05$; Mann-Whitney U test; Figure 5). By contrast, the relative % area of peak 21 ($U = 9$, $Z_{adj} = -2.382$, $P < 0.05$; Mann-Whitney U test; Figure 6) and peak 43 ($U = 13$, $Z_{adj} = -1.958$, $P = 0.05$; Mann-Whitney U test; Figure 7) were larger in lactating females.

In males, the degree of descent of the testes was correlated with the relative amount of peak 21 in winter ($N = 13$, $R = 0.598$, $P < 0.05$; Spearman rank order correlation), with males in breeding condition (i.e., with descended testes) having

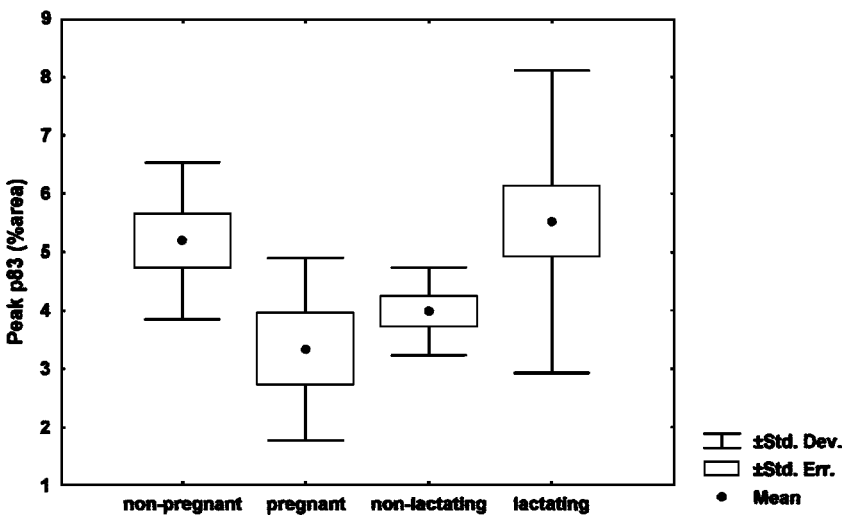


FIG. 5. Differences in % area of peak 83 in female badgers of different reproductive states.

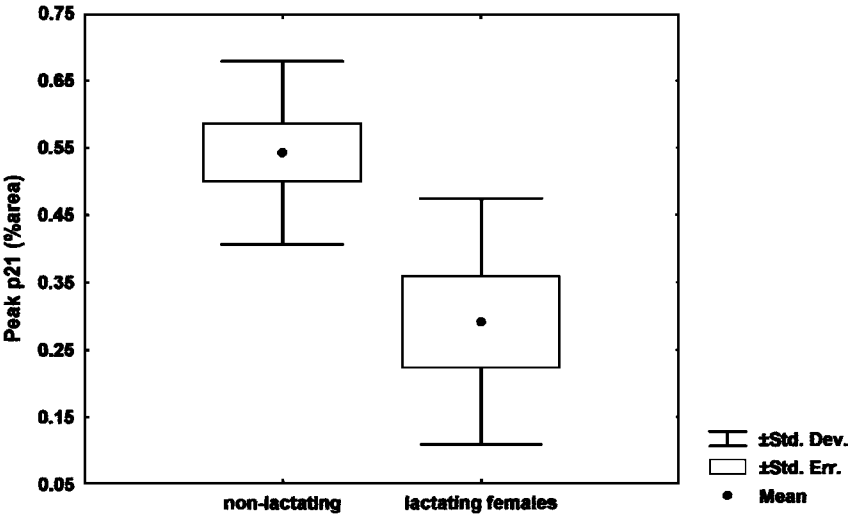


FIG. 6. Differences in % area of peak 21 between lactating and nonlactating females.

a larger % area of peak 21 than males with ascended testes ($U = 3$, $Z_{\text{adj}} = -1.984$, $P < 0.05$; Mann-Whitney U test; Figure 8).

Reproductive condition did not influence average peak numbers in either sex (females: pregnancy: $U = 11$, $Z_{\text{adj}} = -0.216$, $P = 0.829$; lactation: $U = 8$, $Z_{\text{adj}} = -0.494$, $P = 0.621$; males: $U = 8$, $Z_{\text{adj}} = -0.940$, $P = 0.347$).

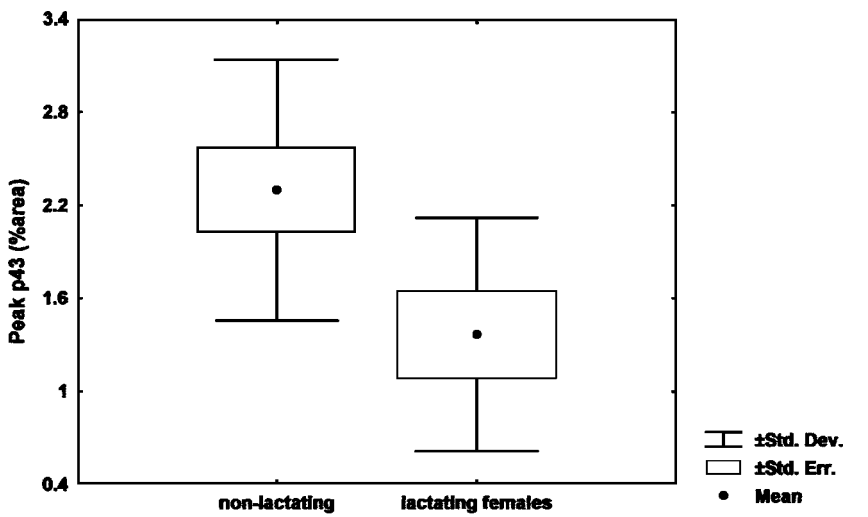


FIG. 7. Differences in % area of peak 43 between lactating and nonlactating females.

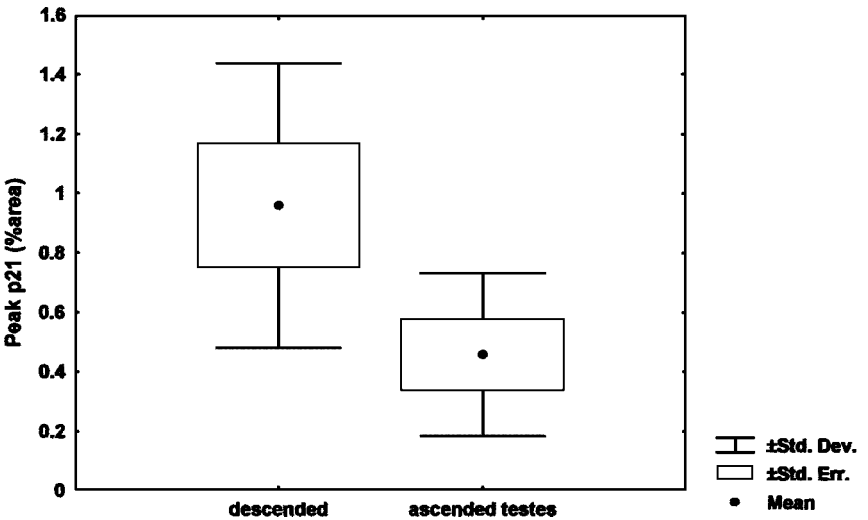


FIG. 8. Differences in % area of peak 21 between males in different reproductive states.

DISCUSSION

Subcaudal gland secretions of badgers are of high chemical complexity. In this study, we identified 21 components that were present in every sample, thus characterizing the species-specific odor. In many mammals, the exact composition of scent secretions has been shown to be of bacterial origin rather than primary gland products (Albone, 1984), and the subcaudal pouches of badgers have been shown to support a rich bacterial flora (Albone et al., 1978). Mutual allo-marking (i.e., two badgers pressing the openings of their subcaudal pouches against each other simultaneously) between group members is believed to facilitate the exchange of pouch bacteria, resulting in a group-specific composition of pouch bacteria, and hence leading to a group-specific smell (Albone et al., 1978; Gorman et al., 1984; Buesching, 2000).

The biological relevance of the chemical differences found in this analysis still remains to be tested. However, in a preliminary study (Buesching and Foster, unpublished data), investigating the behavioral reaction to secretion samples collected from unfamiliar (i.e., unfamiliar samples: from individuals of social groups separated from the focal group by the territories of at least four other groups), neighboring (i.e., familiar samples: from members from the territory adjacent to the territory of the focal group), and own social group (i.e., same group samples: from members of the focal group), it was found that badgers' reactions to the samples were dependent on the degree of their familiarity. Samples (presented as secretion smeared along the rim of a microfuge tube that was pushed completely

into the ground) were provided at one of the sett entrances of the focal sett in full view of an infrared video camera (for methods see Buesching, 2000). Samples were tested in random order [one sample per night; unfamiliar, familiar, same group, blank (i.e., empty microfuge tube), control (i.e., nothing)]. In this experiment, scent-marking activity was influenced by the treatment ($df = 4$, $F = 3.26$, $P = 0.02$; error $df = 45$; ANOVA), with badgers marking most in the presence of unfamiliar samples and least in control nights (unfamiliar > familiar > own > blank > control). Similarly, Kruuk et al. (1984) reported that a captive badger responded differently to the scent of familiar and unfamiliar subcaudal samples. However, it is as yet unclear which cues badgers use for discrimination. Testing the biological relevance of a common group odor remains difficult, as animals might become familiar with the individual odor profiles rather than extrapolate group characteristics (Kruuk et al., 1984).

Besides encoding group membership, the chemical composition of subcaudal gland secretions is also highly individual-specific. We found large interindividual variation in the contents and the relative amounts (i.e., % area) of all components, providing potential for individual recognition by scent (Gorman et al., 1984). Similar individual-specific compositions of scent secretions have been reported in other mustelids [e.g., mink (Brink et al., 1978), stoats (Erlinge et al., 1982), and weasels (Buglass et al., 1991)]. Badgers have been shown to be able to discriminate among samples collected from different individuals (Östborn, 1976; Kruuk et al., 1984), but it is as yet unclear if animals also use this potential for individual recognition under natural conditions. Nevertheless, the consistency of individual characteristics between years (Buesching et al., 2001b) and the use of subcaudal gland secretions as advertisement signals (Buesching, 2000; Buesching and MacDonald, 2001) are further indications that an important part of the information encoded in this signal relates to individuality. The chemical composition of subcaudal secretions is not stable but shows seasonal variation, reflecting the seasonal pattern in the secretion color (Buesching et al., 2002a), and significant variation in the composition of subcaudal gland secretions of individual badgers over time has been reported (Gorman et al., 1984; Buesching et al., 2002b). After correcting for the effect of season, we detected clear sexual differences in the relative amounts of eight of the 21 components present in every profile, again corresponding to the differences in color described elsewhere (Buesching et al., 2001a). Sex-related variation in the composition of scent secretions has been shown in several species [e.g., beavers (Gronneberg and Lie, 1984), capybaras (MacDonald et al., 1984), and peccaries (Waterhouse et al., 1996)] and is believed to serve as sexual advertisement.

Badgers are seasonal breeders (Ahnlund, 1980), and scent-marking activity is highest in winter (mating season) and spring (cub-rearing season) and lowest in autumn (Kruuk, 1978; Buesching, 2000; Buesching and MacDonald, 2001). Additionally, secretions are least concentrated in autumn, which coincides with

the time when badgers store the least amounts of secretion in their subcaudal pouches (Buesching et al., 2002a), indicating the importance of olfactory cues in the context of reproduction.

In both sexes, we detected differences related to reproductive status. Glandular activity is strongly influenced by hormonal status (Ebling, 1977), and many species use chemical signals to advertise reproductive activity (Taylor and Dewsbury, 1990). In several species, advertisement signals have been shown to include fitness-related information [e.g., primates (Pagel, 1994)]. The results of this study indicate that chemical composition of subcaudal gland secretions varies with age and body condition. Similar age-related differences in body odor have been reported, e.g., in humans (Chen and Haviland Jones, 1999). Body condition correlated with the chemical composition of subcaudal gland secretions only in winter and summer, and in female badgers body condition in summer proved to be a good predictor for pregnancy in the following winter (Woodroffe, 1995). As food availability (i.e., earthworms) is lower during these two periods than it is in spring and autumn (Kruuk, 1978), body condition in winter and summer might be a reliable indicator of individual fitness.

The interpretation of the results presented in this study is, however, complicated by several factors. First, the biological relevance of the chemical components in subcaudal gland secretions is unknown. Our analyses concentrated on 21 peaks, that are detectable in every profile, but other, possibly much smaller peaks might be more important to badgers. Second, in complex chemical mixtures, the ratio between a small number of components might be more important than the relative abundance of each individual compound (White and Chambers, 1989). Third, the analytical methods used in this study only detect volatile components soluble in hexane. As badgers possess a functional vomeronasal organ (Lüps and Wandeler, 1993), long chained, nonvolatile molecules might be of equal importance in encoding information (Buesching and Macdonald, 2001).

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GAS-CHROMATOGRAPHIC ANALYSES OF THE SUBCAUDAL GLAND SECRETION OF THE EUROPEAN BADGER (*Meles meles*) PART II: TIME-RELATED VARIATION IN THE INDIVIDUAL-SPECIFIC COMPOSITION

C. D. BUESCHING,[1,*] J. S. WATERHOUSE,[2] and D. W. MACDONALD[1]

[1]*Wildlife Conservation Research Unit*
Department of Zoology
University of Oxford
South Parks Road, Oxford, OX1 3PS, UK

[2]*Environmental Sciences Research Centre*
Anglia Polytechnic University
East Road, Cambridge, CB1 1PT, UK

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Abstract—Individuality in body odors has been described in a variety of species, but studies on time-related variation in individual scent are scarce. Here, we use GC-MS to investigate how chemical composition of subcaudal gland secretions of European badgers (*Meles meles*) varies over days, seasons, and from year to year, and how secretions change with the length of time for which they are exposed to the environment. Samples were divided into subsamples—one was frozen immediately and the remaining ones frozen after 2, 6, 12, 24, and 48 hr, respectively—and many individual-specific characteristics of the scent-profiles remained stable over time. However, two components were negatively correlated with time, thus providing the possibility to determine the age of scent marks. The low variation found in scent profiles of samples collected from the same individual three days apart showed that the individual-specific scent is a true characteristic of the respective badger, and that trapping and subsequent sampling have little effect on the composition of subcaudal gland secretions. Long-term variation (i.e., over one year) in individual subcaudal scent profiles is not continuous, but periods of relative stability are followed by periods of rapid change, that can be related to badger biology. Annual variation between samples collected from the same individuals in winter 1998 and winter 1999, and in spring 1998 and spring 1999 was lower than seasonal variation. Therefore, the results of this study indicate the potential of an individual-specific scent

*To whom correspondence should be addressed.

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signature in the subcaudal gland secretions of badgers evidencing that individual recognition is of high importance in this species.

Key Words—Badger, body odor, individuality, individual variation, Mustelidae, chemical communication, scent-marking, semiochemistry, subcaudal gland, temporal variation.

INTRODUCTION

Individuality in body odors has been described in a variety of mammals (review in Brown and Macdonald, 1985). However, few studies examine how individual scent varies over time [e.g., brown hyaenas (Mills et al., 1980)]. In this study, we investigated how chemical composition of subcaudal gland secretions produced by individual badgers varies over time and how secretion changes with the length of time it is exposed to the environment.

The subcaudal gland of the European badger (*Meles meles*) consists of a pouch lined with several layers of apocrine and sebaceous cells (Stubbe, 1971) that opens in a 20 to 80-mm-wide slit between the anus and the root of the tail. Both sexes possess the gland and use its pastelike secretion for scent-marking. Subcaudal gland secretions contain mainly long-chained carboxylic acids, proteins, and water (Gorman et al., 1984). The characteristic smell is generated largely by bacteria in the pouch metabolizing the primary gland products (Kruuk et al., 1984). The color and amount of the secretion have been shown to follow a seasonal pattern and are influenced by sex and fitness-related parameters (Buesching et al., 2002a). Analytical gas chromatography and mass spectrometry studies proved that chemical composition is highly individual-specific (Gorman et al., 1984; Buesching et al., 2002b). Only 21 of the full complement of approx. 110 chemical components are present in every sample (average number of chemical components per sample $= 33.4 \pm 8.05$). Composition varies between seasons and between different badger groups, and is dependent on sex, age, body condition, and reproductive status (Buesching et al., 2002b). However, whereas visual and acoustic signals are received the moment they are emitted, and can thus, be matched immediately to other characteristics of the sender, chemical signals are persistent and are often used for delayed communication, implying that the receiver usually encounters scent marks only after the sender (i.e., the marker) has left the area. Therefore, to ensure effective communication and individual recognition, information-encoding components of chemical signals have to be reasonably stable over time in two respects: (1) composition of scent marks has to resist environmental influences to avoid loss of encoded information; and (2) composition of secretions produced by badgers has to remain similar over some period of time to enable individual recognition and the matching of individuals to scent marks.

In this paper, we report on how chemical composition of subcaudal gland secretions of individual badgers varies over days, seasons, and years; how sampling

affects individual scent composition; and how secretions change with the length of time they are exposed to the environment.

METHODS AND MATERIALS

Study Animals and Collection of Samples. All samples were collected from a badger population in Wytham Woods, Oxfordshire, England (ordnance survey reference: SP462080; for details about the study site see Kruuk, 1989; for details about the badger population see Macdonald and Newman, 2001) between January 1998 and June 1999.

For this study, badgers were trapped four times per year (winter: January/February; spring: May/June; summer: July/August; and autumn: November). For methodological details and classification of individual-specific parameters see Buesching et al. (2002b).

Gas-Chromatographic Analyses. For methodology see Buesching et al. (2002b).

Statistical Analyses of GC Profiles. For a detailed description of the methodology see Buesching et al. (2002b). In this study, the 21 peaks shared by every individual (i.e., present in every profile) are referred to as "shared peaks," whereas peaks that could not be detected in every profile are referred to as "rare peaks" (for details see Buesching et al., 2002b).

Variation in scent profiles of the same individual over time (i.e., between trapping events) was expressed as percentage change (%change) and calculated according to the following formula:

$$\%\text{change}_{\text{peak } x} \text{ in Interval } x = 100 * (\%\text{area}_{\text{peak } x} \text{ at trapping } 1 - \%\text{area}_{\text{peak } x} \text{ at trapping } 2)/(\%\text{area}_{\text{peak } x} \text{ at trapping } 1)$$

where Interval 1 is winter (= trapping 1)–spring (= trapping 2) 1998, Interval 2 is spring (= tr.1)–summer (= tr. 2) 1998, Interval 3 is summer (= tr. 1)–autumn (= tr. 2) 1998, Interval 4 is autumn 1998 (= tr. 1)–winter (= tr. 2) 1999, Interval 10 is winter 1998 (= tr. 1)–winter 1999 (= tr. 2), and Interval 20 is spring 1998 (= tr. 1)–spring 1999 (= tr. 2).

A measure for the change of the overall profile (= change rate) was obtained by calculating the mean % change over all peaks. Statistical analyses were carried out with the software program Statistica for Windows (Release 4.5, StatSoft Inc., 1993).

Changes of Secretion in the Environment. To investigate changes in the chemical composition of subcaudal gland secretions related to the length of time secretions had been exposed to the environment before freezing, six samples from three females and three males were collected on June 3, 1999. Each sample was divided into six equal-size subsamples, one of which was frozen immediately and

the others were left outside in the shade for 2, 6, 12, and 48 hr, respectively, before freezing. All samples were stored at −70°C for five months until further analysis.

Short-Term Changes. To assess how individual scent profiles change within a matter of days and to examine how trapping and subsequent secretion-sampling affect scent profiles of individuals, we compared the chemical composition of subcaudal gland secretions from one male and one female collected on the day of first capture May 31, 1998 and three days later June 2, 1998. For both animals, change rates and % change of all peaks were calculated and compared with the respective values from intervals 1 and 2. Secretions were frozen immediately after sampling and stored at −70°C for a period of five months prior to GC analysis.

Long-Term Changes. To evaluate long-term changes in the chemical composition of secretions from individual badgers, we analyzed scent profiles from four males and four females collected during six consecutive trappings between January, 1998 and January, 1999. All samples were frozen immediately and stored at −70°C for a maximum of eight months prior to GC analysis. To explore whether interindividual differences described in earlier studies (Gorman et al., 1984; Buesching et al., 2002b) are consistent over season, we performed a MANOVA (corrected for sex and season) comparing intra- and interindividual variability of the % area of all shared peaks.

We compared % change of all peaks and change rates over the whole profile during each interval to investigate whether chemical composition of gland secretions changes continuously over the year or whether periods of relative stability are followed by periods of rapid change. To examine if individual profiles vary at random over the course of one year, or if variation follows a regular seasonal pattern (i.e., profiles obtained from the same individual in the same season but in different years are more similar than profiles obtained from the same individual in different seasons within the same year), we compared scent-profiles from three females and four males from winter 1998 and winter 1999, and from seven females and four males from spring 1998 and spring 1999.

RESULTS

In the 112 samples analyzed for this study, we found 110 different chemical components, of which 21 were shared by every individual (i.e., shared peaks), the remainder being "rare peaks" (for details see Buesching et al., 2002b). Since, in several profiles, retention times for peaks p77, p78, and p79 were partly overlapping and thus could not be determined accurately, % areas of these three peaks were combined for all further analyses.

Changes of Secretion in the Environment. Although chemical composition of subcaudal gland secretions changed over time, no pattern in the presence or absence of peaks could be established, and the number of peaks per profile was not

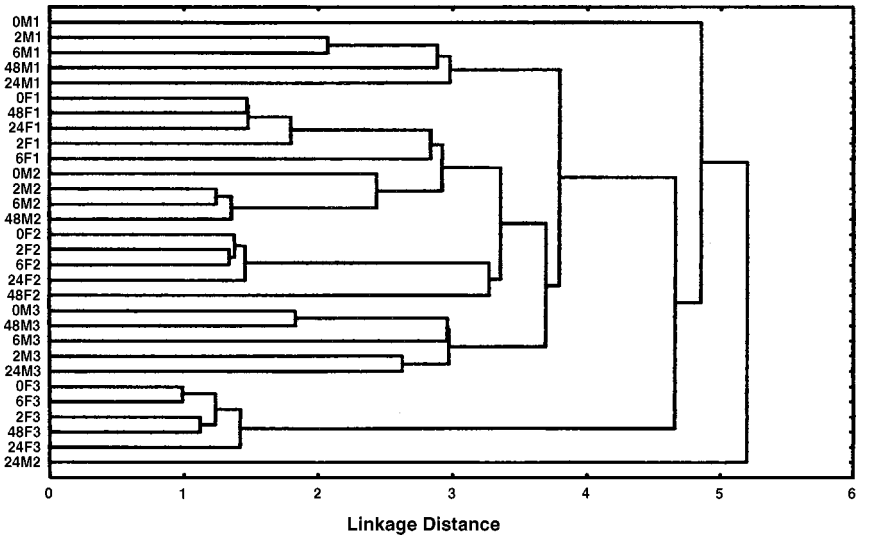


FIG. 1. Cluster analysis (including all peaks in the first part of the profiles up to p68) indicating that individuality is encoded mainly in the first part of the profile, which is stable over time ($M_{1,2,3}$ = male; $F_{1,2,3}$ = female; at intervals of 0, 2, 6, 24, and 48 hr).

correlated to the time span, for which samples were left in the environment before freezing ($P > 0.05$; Spearman rank correlation). Cluster analysis (single Euclidian distance, unweighted pair group average) revealed that the first part of the profiles (including all peaks prior to peak p68) was highly individual-specific and stayed relatively stable over time. Thus, samples of the same individual clustered closely together (Figure 1). However, cluster analysis including all peaks did not result in individual-specific clusters.

For statistical reasons, more detailed analyses were restricted to the shared peaks. Peak p90 ($N = 30$, $R = -0.378$, $P < 0.05$) and peak p91 ($N = 30$, $R = -0.395$, $P < 0.05$) proved to be negatively correlated with the time, for which the samples were left outside before freezing (Figure 2).

Short-Term Changes. As apparent from Figure 3, there is remarkably little change in individual profiles over short time periods. Comparison of secretions from the same individuals collected three days apart revealed that on the day of recapture % area of all shared peaks was within 33% (average % change for all peaks $14.66 \pm 13.99\%$; range: 0.258–32.526%) of its original value in both animals, and 95% of the rarer peaks were present in both of the respective profiles (Figure 3). Change rates were smaller among days than for the previous period, interval 1 ($U = 1126.5$, $Z_{adj} = -5.809$, $P < 0.001$; Mann-Whitney U test) and the following period, interval 2 ($U = 972$, $Z_{adj} = -6.319$, $P < 0.001$; Mann-Whitney U test).

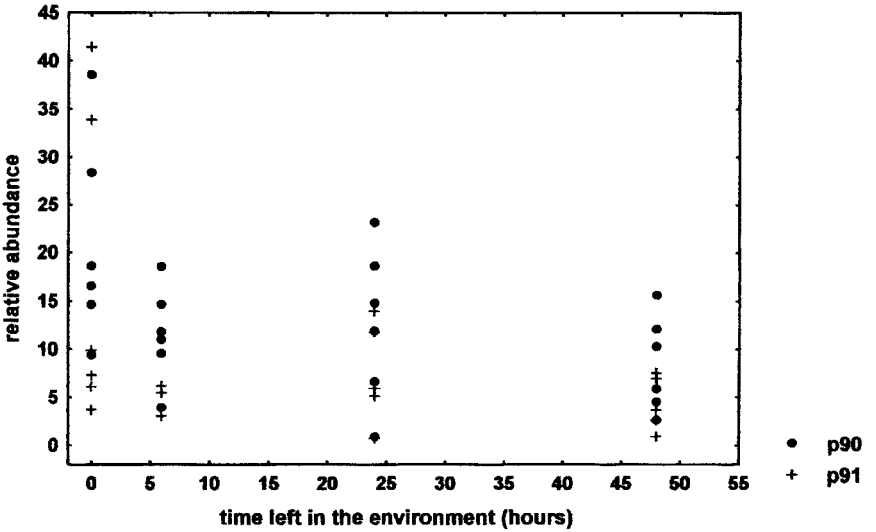


FIG. 2. Relative % area of peaks p90 and p91 are negatively correlated with the age of the scent-mark.

Long-Term Changes. Our results comparing intra- and interindividual variability in % area of all shared peaks indicate that approx. 42% of the shared peaks (8 of 19) show less variability within samples of the same individual (collected in different seasons) than between individuals (Table 1), indicating the existence of a unique scent signature in badgers. Comparative analyses of secretions collected from the same individuals in different seasons showed that change rates differ between sexes (Figure 4). Subcaudal scent profiles of female badgers change most between spring and summer (change $\text{rate}_{\text{interval2}} = 173.8 \pm 368.4\%$) and between autumn and winter (change $\text{rate}_{\text{interval4}} = 251.2 \pm 521.4\%$), but remain comparatively stable during the remaining intervals (change $\text{rate}_{\text{interval1}}$: $71.9 \pm 94.5\%$; change $\text{rate}_{\text{interval3}}$: $80.4 \pm 85.0\%$; $N = 4$). Thus, change rates in interval 1 appeared lower than in interval 2 ($U = 2415.5$, $Z_{\text{adj}} = -1.741$, $P = 0.08$) and were significantly lower than in interval 4 ($U = 1122$, $Z_{\text{adj}} = -1.935$, $P = 0.05$; Mann-Whitney U test). In contrast, male profiles show most pronounced changes between summer and autumn (change $\text{rate}_{\text{interval3}}$: $156.5 \pm 279.1\%$) and between autumn and winter (change $\text{rate}_{\text{interval4}}$: $121.4 \pm 204.3\%$) and show little change between winter and spring (change $\text{rate}_{\text{interval1}}$: $67.7 \pm 116.2\%$) and spring and summer (change $\text{rate}_{\text{interval2}}$: $99.6 \pm 328.5\%$; $N = 4$). Therefore, male change rates were lower in interval 1 than in interval 3 ($U = 1988$, $Z_{\text{adj}} = -3.316$, $P < 0.001$) and interval 4 ($U = 1722$, $Z_{\text{adj}} = -2.019$, $P < 0.05$) and also lower in interval 2 than in interval 3 ($U = 2197$, $Z_{\text{adj}} = -2.546$, $P < 0.05$; Mann-Whitney U test) (Figure 4).

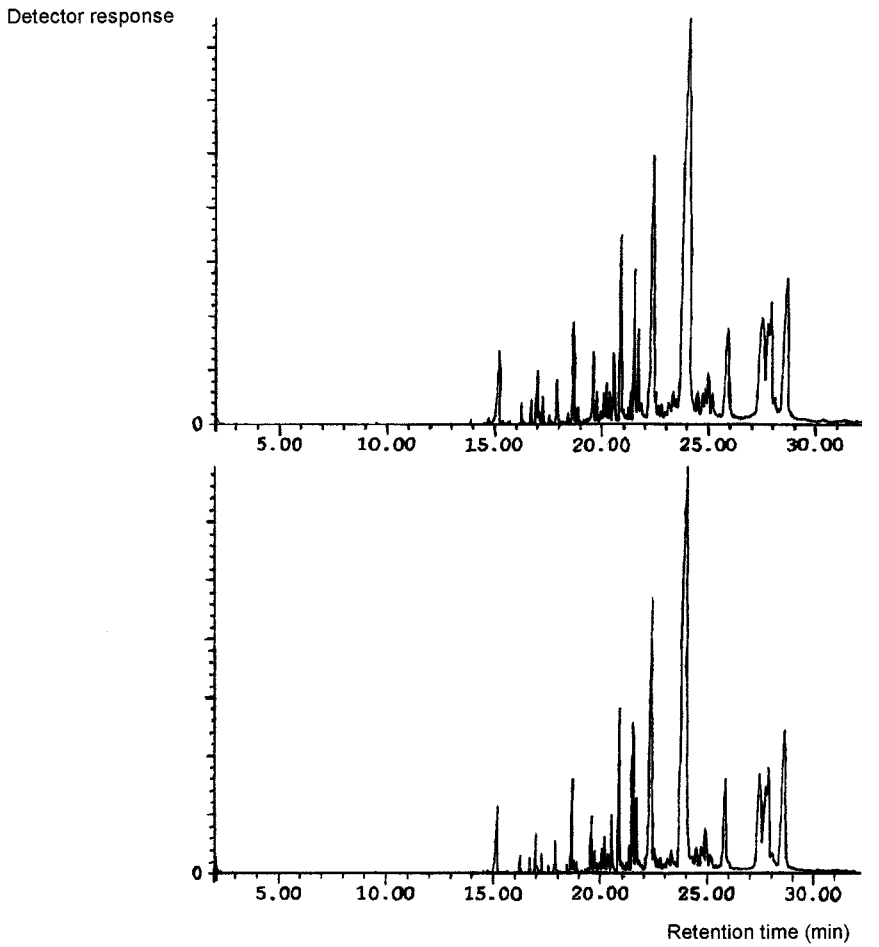


FIG. 3. Scent profiles of two samples from male 623 collected three days apart (the upper profile corresponds to the sample collected first).

To asses the pattern of change in individual peaks, we conducted a MANOVA using interval and sex as factors (Table 1). These analyses revealed that not all peak values change continuously, but that the rate of change differs between intervals. In peak p91 and p95, the pattern of seasonal change significantly differs between males and females (Table 2). Although the chemical composition of subcaudal gland secretions considerably varies with season, chemical composition of subcaudal gland secretions from the same individual collected in the same season of two different years is remarkably similar (Figure 4). On average, female composition changes 83.6 $\pm$ 92.3% between years in winter, but only 36.8 $\pm$ 38.3% in spring,

TABLE 1. MANOVA RESULTS ILLUSTRATING INTER- AND INTRAINDIVIDUAL VARIABILITY IN %AREA OF 21 SHARED PEAKS[a]

Peak	$F_{(df\,1,2)}$	P
p5	2.617	0.059
p11	0.706	0.668
p13	1.928	0.140
p21	15.398	<0.001*
p24	11.229	<0.001*
p29	2.101	0.112
p43	15.827	<0.001*
p47	1.549	0.230
p48	26.1126	<0.001*
p50	4.226	0.010*
p56	1.322	0.310
p68	2.618	0.059
p75	1.027	0.455
p77–79	8.129	<0.001*
p83	3.130	0.033*
p89	2.302	0.087
p90	1.431	0.268
p91	2.456	0.723
p95	3.850	0.015*

[a] ($F_{1,2} = 7.14$).
*$P < 0.05$ indicates significantly lower intraindividual variability.

whereas male secretions change approx. $57.9 \pm 78.7\%$ between years in winter and $51.5 \pm 59.5\%$ in spring. In neither sex do annual winter and spring change rates differ from seasonal changes in interval 1 (winter: females: $F = 0.868$, $P = 0.768$, males: $F = 0.892$, $P = 0.765$; spring: females: $F = 0.728$, $P = 0.394$, males: $F = 0.143$, $P = 0.706$; F test). However, whereas in females both annual change rates are smaller than the average percentage change in interval 2 (winter: $F = 5.148$, $P < 0.05$; spring: $F = 15.106$, $P < 0.001$; F test) and interval 4 (winter: $F = 12.452$, $P < 0.001$; spring: $F = 23.964$, $P < 0.001$; F test), but not interval 3 (winter: $F = 0.006$, $P = 0.936$; spring: $F = 1.224$, $P = 0.269$; F test), in males annual change rates in winter and spring are smaller than seasonal change in interval 3 (winter: $F = 7.743$, $P < 0.01$; spring: $F = 11.003$, $P < 0.001$; F test) and interval 4 (winter: $F = 4.140$, $P < 0.05$; spring: $F = 5.264$, $P < 0.05$; F test), but not interval 2 (winter: $F = 1.422$, $P = 0.234$; spring: $F = 2.060$, $P = 0.152$; F test) (Figure 4). On average, 84.2% of all rare peaks were shared by the pairs of winter and spring profiles of the same individual recorded in different years.

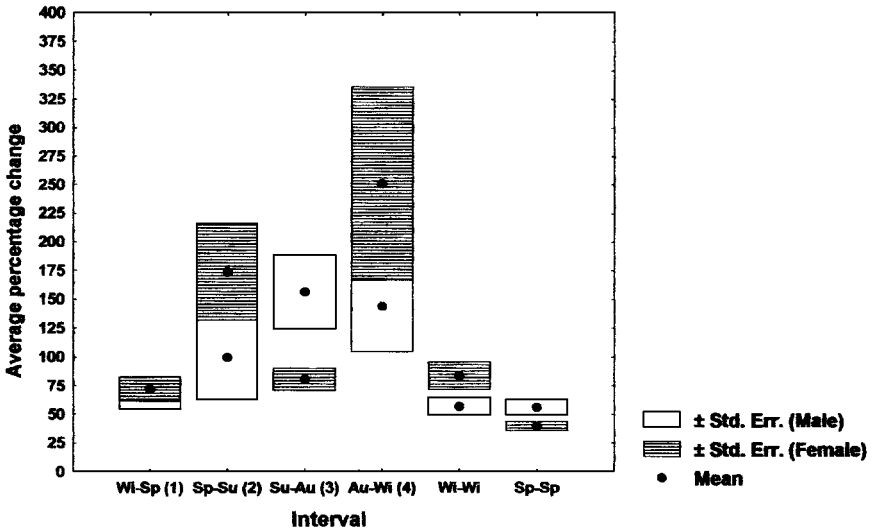


FIG. 4. Long-term average % change in gas chromatography profiles of the same individual.

DISCUSSION

Badgers rely heavily on their chemical senses and use subcaudal gland secretion to mark their environment and conspecifics (Kruuk, 1989). Subcaudal gland secretions contain mainly long-chain, nonvolatile carboxylic acids that are released slowly (Gorman et al., 1984). Thus, scent marks can be detected for some period after they have been deposited by the sender (i.e., the marking individual) and are, therefore, long-term signals suitable for delayed communication. Our results show that chemical composition remains relatively stable over time, when secretions are exposed to the environment. These findings are in concordance with Gorman et al. (1984), who stated that secretions smeared on glass slides and left in the lab for a period of five days did not change their composition appreciably.

Subcaudal scent-marking is believed to play an important role in territorial defense (Kruuk et al., 1984). By marking the territory with the scent of its owners, an asymmetry of context can be established between territory owner and intruder, as only residents will have had time for extensive scent-marking activity (Gosling, 1982). Thus, it is important that individual-specific information encoded in scent marks be resistant to environmental influences to enable matching scent marks to individuals. However, although chemical composition remained relatively stable over time, our results also show that % area of peaks p90 and p91 are negatively correlated with the time subcaudal gland secretions are left in the environment before freezing. Similar oxidation effects of scent marks have been described in

TABLE 2. MANOVA RESULTS ILLUSTRATING SPECIFIC EFFECTS OF SEX AND INTERVAL ON CHANGE RATES OF DIFFERENT PEAKS[a]

	Main effect "interval"		Main effect "sex"	
Peak	$F_{(df\,1,2)}$ 3,21	P	$F_{(df\,1,2)}$ 1,21	P
p5	2.060	0.133	<0.001	0.998
p11	2.723	0.070[a]	0.963	0.338
p13	4.557	0.013[a]	1.723	0.203
p21	0.546	0.656	0.032	0.860
p24	1.414	0.267	0.833	0.372
p29	1.301	0.300	0.221	0.643
p43	0.446	0.723	0.667	0.423
p47	4.074	0.020[a]	2.509	0.128
p48	3.470	0.034[a]	1.447	0.242
p50	0.749	0.535	0.731	0.402
p56	8.518	0.001[a]	1.711	0.205
p68	2.047	0.138	1.884	0.184
p75	3.289	0.041[a]	0.846	0.368
p77–79	0.401	0.754	1.198	0.286
p83	0.667	0.581	1.478	0.237
p89	0.051	0.984	1.124	0.301
p90	0.870	0.472	1.854	0.188
p91	26.953	<0.001[a]	19.529	<0.001[a]
p95	0.660	0.586	4.637	0.043[a]

[a]$P < 0.05$ is considered to be significant.

aardwolves (*Proteles cristatus*) (Sliwa, 1996), peccaries (Sowls, 1984), and brown hyaenas (Hyaena brunnea) (Mills et al., 1980), where secretion color changes over time of exposure to the environment. Klipspringers (*Oreotragus oreotragus*) have been shown to adapt their behavioral response to the age of the scent marks they encounter (Roberts, 1998). Including information about the age of the scent mark will help the receiver to decide if the scent mark is still relevant; this also benefits the sender by alerting receivers to recent scent marks. Information on the age of scent marks is especially important in the context of reproduction. In badgers, it has been shown that subcaudal secretions may include information about age, body condition, and reproductive status of the marking individual (Buesching et al., 2002a,b). Thus, highly increased marking frequencies during the reproductive season (Buesching and Macdonald, 2001) will provide accurate information about individual-specific parameters of the sender and will pinpoint locations that have recently been used by this animal.

Subcaudal samples collected three days apart from the same individual varied little in their chemical composition, indicating that trapping and sampling

have little effect on the individual-specific composition of badger subcaudal gland secretions. Despite the small sample size, these results reinforce that subcaudal scent is mainly determined by factors intrinsic to the badger and is, thus, a true characteristic typical for the respective individual. However, glandular activity and composition of gland secretions are under strong hormonal control (e.g., Ebling, 1977; Balakrishnan et al., 1984; Müller-Schwarze, 1983) and are also dependent on individual-specific parameters, such as sex, age, body condition, and dominance status (e.g., Atkeson and Marchington, 1982; Schilling and Perret, 1987; Buesching et al., 2002a,b). Additionally, chemical composition is often influenced by food sources. Thus, individual body odor is usually liable to changes over time. In badgers, color and volume of subcaudal gland secretions have been shown to vary with season and to depend on sex, age, body condition, and reproductive status (Buesching et al., 2002a). Gorman et al. (1984) showed that chemical composition of subcaudal gland secretions changes over time, although individuals can be recognized for a period of 80–160 days (Kruuk et al., 1984). Similarly, anal sac secretions of several mustelid species have been shown to be individual-specific and stable over time. For example, individual mink show similar patterns throughout the year (Brink et al., 1978), and in stoats, anal gland secretions of individuals remain similar for at least one month (Erlinge et al., 1982). In the individual-specific composition of subcaudal gland secretions of badgers, variation is not continuous, but periods of relative stability are followed by intervals of rapid change. In both sexes, change is lowest during the reproductive period between January and May, coinciding with a peak in scent-marking activity (Buesching, 2000; Buesching and Macdonald, 2001). Recognizing individuals during the breeding season is especially important in the context of mate choice and avoidance of incest. In badgers, dispersal is rare (Woodroffe and Macdonald, 1992), and females tend to mate with most males in their own social group (Macdonald et al., unpublished data), yet the degree of relatedness among members of the same social group is lower than expected (Evans et al., 1989).

Highest % change rates occur between autumn and winter. During this time, badgers switch their main food source from earthworms to cereals and nuts, and their metabolism changes from catabolic to anabolic (Neal, 1986). In autumn, scent-marking activities are lowest (Buesching, 2000; Buesching and Macdonald, 2001), reflecting a relative annual minimum in badger social behavior in general (Stewart, 1997).

The high degree of similarity in secretions of the same individual collected in the same season of two succeeding years indicates that variation in the chemical composition is not random, but highly individual-specific. Additionally, over 40% of all shared peaks showed less intra- than interindividual variability over the course of one year. In our opinion, these results, although derived from a relatively small sample size, indicate the existence of a unique individual-specific scent signature

in the subcaudal gland secretions of badgers. Thus, we suggest that, although badgers are often considered as primitively social mammals (Kruuk, 1989) due to their plasticity in social organization (Woodroffe and Macdonald, 1992) and their lack of truly cooperative behavior (Stewart, 1997), individual recognition is of high importance.

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DUFOUR GLAND CONTENTS OF ANTS OF THE *Cataglyphis bicolor* GROUP

OYA A. GÖKÇEN,[1] E. DAVID MORGAN,[1,*] FRANCESCA R. DANI,[1] DONAT AGOSTI,[2] and RÜDIGER WEHNER[2]

[1]*Department of Chemistry*
Keele University, Keele
Staffordshire, England ST5 5BG

[2]*Zoologisches Institut der Universität Zürich*
Winterthurerstrasse 190
CH-8057 Zürich, Switzerland

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Abstract—The species of desert-dwelling ants of the *Cataglyphis bicolor* (Hymenoptera: Formicidae) group are difficult to distinguish by morphological features. Analysis of the secretion from the Dufour glands of workers of a number of colonies was undertaken to see if it provided a clear test of species. Linked 6c-ms showed in all samples straight and branched-chain alkanes, linear alkenes, ketones, aldehydes, acetates, and a group of C_{22} to C_{28} esters not previously identified in this genus. Contents of the Dufour glands of *C. savignyi* from Tunisia and Egypt were similar, and comprised straight and branched-chain alkanes, alkenes and small amounts of esters. *C. bicolor* from Tunisia contained compounds similar to *C. savignyi* but was distinguished from the latter by larger amounts of the esters. The major compound in the glands of *C. viaticus* was tridecane, in contrast to the pentadecane of other species. It also contained a branched alkane, 3-methyltridecane as a major component. Branched-chain esters and a wide variety of acetates were also found in this species. *C. diehlii* had a limited range of compounds, with branched alkanes almost completely absent and high proportions of pentadecene and dodecyl acetate. *C. bombycinus*, a sympatric species, but recognized as not belonging to the *bicolor* group by its different mandibular gland substances, was notable in having butanoate esters in its Dufour glands. Despite these differences among species, both the great variability of individuals from a single colony and the among between conspecific colonies make species diagnosis from a few individuals difficult, in contrast with postpharyngeal glands, which, as recently reported, give a clearer indication of species.

Key Words—Hymenoptera, Formicidae, Formicinae, exocrine secretion, hydrocarbons, esters, species specificity, species diagnosis.

*To whom correspondence should be addressed.

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INTRODUCTION

The ant genus *Cataglyphis* includes more than one hundred named taxa, with others yet undescribed, and is distributed in the Old World from Mauritania to the Gobi Desert. Almost all species thrive in open habitats, including deserts and dry salt plains. Because of their thermophilic and desert-dwelling behavior, they have been the subject of intensive research in neuroethology (Wehner, 1994) and physiology (Lighton and Wehner, 1993). Much of this work was carried out on "*Cataglyphis bicolor*", but during the course of this work it became apparent that this "*C. bicolor*" was either one species which varied slightly in external appearance from place to place or it was a group of closely similar species, and these were tentatively referred to as forms a, b, and c in Wehner et al., (1983).

Cataglyphis are unique among formicine ants in that the morphology of the male genitalia is diverse, making this genus easily distinguishable from others. However, anatomical similarities make the classification difficult at the species level. Because the morphological characters are lacking, neither can the species be diagnosed sufficiently nor can the phylogenetic relationships among species be easily inferred (Agosti, 1990). The work of Santschi (1929), who spent four decades in Tunisia concentrating on *Cataglyphis*, left a tangle of specific and subspecific names and synonyms. The work of Agosti (1990) supported by chemical studies of exocrine secretions of the groups (Keegans et al., 1992) and isozyme and *m* DNA analyses (Ready and Agosti, unpublished) have made possible the identification of some species of the group.

The Dufour gland is present in all females and workers of aculeate Hymenoptera, and although it has been shown to have a function (presumably secondary) in some individual species or genera, it has no known common function. Nevertheless, its contents have been found to be species-specific in many studies where closely related species have been compared. Usually the gland contains a mixture of linear hydrocarbons, terpenes, or oxygenated alkyl compounds.

Some chemotaxonomic studies have already been carried out on the Dufour gland contents of *Cataglyphis* species. The Dufour glands of *C. savignyi* from Egypt were shown to contain straight chain saturated and unsaturated hydrocarbons from C_{11} to C_{23}, together with some branched hydrocarbons with methyl groups at 3-, 5-, and 7-position. Oxygenated compounds (e.g., 2-ketones, 1-alcohols and branched chain aldehydes) were identified in trace quantities (Ali et al., 1988). Hefetz and Lenoir (1992) analysed Dufour glands of six *Cataglyphis* species from Israel, Tunisia, Spain, and the South of France. The major common feature was saturated hydrocarbons ranging from C_{11} to C_{19}. Minor amounts of corresponding alkenes and some oxygenated components in trace amounts were also observed. The main differences among species were expressed in the identity of the major components and the relative amounts of all components present in the secretion.

Our work, previously described for 10 species of *Cataglyphis* collected in Syria and Tunisia, showed that those species could be distinguished by their Dufour gland contents (Keegans et al., 1992). Five belonging to the bicolor group were similar. *C. niger* and *C. nodus* had pentadecane, tridecane, heptadecane, tetradecane, and tridecane in decreasing amounts as the major hydrocarbons. However, *C. niger* had equal quantities of 2-pentadecanone and 2-heptadecanone, whereas *C. nodus* had more 2-pentadecanone than 2-heptadecanone. The two types from Tunisia, called at that time *C. bicolor*-1 and *C. bicolor*-2, were similar to *C. niger* and *C. nodus* but also showed some differences. *C. bicolor*-1 had more tridecane (the major compound) and dodecanol than the other species but less pentadecanone than *C. niger* or *C. nodus*. The most obvious difference in *C. bicolor*-2 was the low amount of oxygenated compounds in its gland. *C. isis*, also from the bicolor group, was distinguished from the other four by the large amounts of dodecanol and tridecane it contained (Keegans et al., 1992).

We also discovered that the *bicolor* group has a distinctive feature in that the species examined all contain 2-methyl-1-hexanol, a new substance to exocrine secretions, as the major substance in their mandibular glands (Agosti et al., 1996). This substance was not found in any species outside the *bicolor* group.

For the present work, colonies of the *C. bicolor* group were collected at various sites in Tunisia and Egypt, identified only by a code (which referred to some morphological character, Table 1) or by the name of the place of collection. Samples of Dufour glands were taken from workers for chemical analysis. The code names were united with the new species names (*bicolor*, *diehlii*, *savignyi* and *viaticus*) derived from morphological examination of the male genitalia, after the analyses were finished. We describe the chemical examination and statistical analysis of the results of the analysis of Dufour glands here as they relate to these species and one more sympatric species, *C. bombycinus*, which was different and belongs to another *Cataglyphis* group.

MATERIALS AND METHODS

The ants were collected at sites in Tunisia and Egypt by D. Agosti during 1992. For location, see Wehner et al., (1994). The nests were brought live to Zürich where the workers were dissected. The Dufour glands were sealed in glass capillaries for transport to Keele as described by Morgan (1990). They were analyzed there by linked gas-chromatography-mass spectrometry (GC-MS), by the method of Morgan and Wadhams (1972).

The GC-MS was performed with a Hewlett-Packard 5890 gas chromatograph linked to a 5970B Mass Selective Detector, set to monitor m/z 35–350. The system was controlled by an HP Series 300 computer with HP 59970C ChemStation software. A fused silica capillary column (12 m $\times$ 0.32 mm; 0.25 μm) of 5%

TABLE 1. COLLECTION AREAS OF THE *Cataglyphis bicolor* GROUP SAMPLES IN NORTH AFRICA

Sample name*	Collection area†	Species	Country
bdw t1 10a	Transect 1-location 10	*savignyi*	Tunisia
bdw t1 10b	Transect 1-location 10	*savignyi*	Tunisia
bdw t1 10c	Transect 1-location 10	*savignyi*	Tunisia
bdw t3 1a	Transect 3-location 1	*savignyi*	Tunisia
bdw t3 2a	Transect 3-location 2	*savignyi*	Tunisia
bdw t3 2b	Transect 3-location 2	*savignyi*	Tunisia
bdb t3 1b	Transect 3-location 1	*bicolor*	Tunisia
bdb t3 2c	Transect 3-location 2	*bicolor*	Tunisia
bdb t3 2e	Transect 3-location 2	*bicolor*	Tunisia
bdb t3 3d	Transect 3-location 3	*bicolor*	Tunisia
bdb t3 6a	Transect 3-location 6	*bicolor*	Tunisia
bdb t3 7c	Transect 3-location 7	*bicolor*	Tunisia
bb t3 6a	Transect 3-location 6	*viaticus*	Tunisia
bb t3 7d	Transect 3-location 7	*viaticus*	Tunisia
bb t3 8c	Transect 3-location 8	*viaticus*	Tunisia
bb t3 10a	Transect 3-location 10	*viaticus*	Tunisia
Gafsa-bom	Gafsa	*bombycinus*	Tunisia
Kebili-bom	Kebili	*bombycinus*	Tunisia
Diehlii	Metlaoui	*diehlii*	Tunisia
Siwa 3	Siwa	*savignyi*	Egypt
Siwa 6	Siwa	*savignyi*	Egypt
Gizeh 3 31	Gizeh	*savignyi*	Egypt
Luxor	Luxor	*savignyi*	Egypt
Marsah Matruh	Marsah Matruh	*savignyi*	Egypt
Dakhla	Dakhla	*savignyi*	Egypt

*Samples were assigned to groups by the following characters: bdw, Large workers with head and alitrunk dark red to black, legs concolor to black; bdb, Alitrunk and dorsum of petiole with few white or yellowish, short erect hairs, which are less than half the length of the black, erect hairs on the occiput; and bb, Large workers with head and alitrunk bright red, legs sometimes slightly darker.

†For map, see Figure 1, also Wehner et al. (1994) or Agosti et al. (1996).

phenyl −95% methylsiloxane (non-polar phase) (SGE Milton Keynes) was used for chromatography. The samples were heated in the injector at 200°C for 3 min before crushing the glass capillary. The column was initially at 30°C for 2 min, then heated at 8°C min^{-1} to 250°C, and held at that temperature, to give a total run time of 40 min. The split valve was closed for the injection and opened 30 sec after injection.

Identification of the compounds was made with the aid of The Wiley- NIST mass spectral library (1994 version), and collections of spectra made in the laboratory. Final identification was made, wherever possible, by comparing retention times and mass spectra with commercially available materials (linear alkanes,

2-alcohols, and 2-alkanones) or in the cases of 2-tridecyl acetate, dodecyl decanoate, dodecyl tetradecanoate, and butyl oleate they were synthesized in the laboratory. Esters were prepared on a small scale by the method of Attygalle et al., (1987). For quantitative analysis, peak areas for each component in the chromatogram were determined by computer integration, and the percentage of each substance in the gland was calculated. For each compound, average percentage value and standard deviation were calculated for each colony and for each group of colonies identified by the same code. In the *t* test, data transformed by arcsin $[\sqrt{(\%/100)}]$ were used (Sokal and Rohlf, 1995).

RESULTS

Since the work was begun blind, a few mandibular glands of workers from each of the 25 colonies provided were examined first by GC-MS. In 23 out of 25 colonies, samples all contained 2-methyl-1-hexanol (Agosti et al., 1996), and these colonies were all placed in the *bicolor* group based on this evidence. Only the Gafsa and Kebili samples did not contain 2-methylhexanol; they both contained citronellol and citral (c.f. Keegans et al., 1992), and these colonies were assigned to the *bombycinus* group.

Altogether, 7057 chromatographic peaks from Dufour glands were identified and quantified for 195 workers from 25 colonies. For each of these colonies, the mean values of the area for each peak were calculated together with their standard deviations.

Table 1 gives the code names of the samples, the number of specimens analyzed per colony, their place of origin, and the species names assigned. The location of the sample sites are given in Figure 1.

C. savignyi, Tunisia (bdw)

Five colonies were analyzed. In each specimen but 3, the major compounds, beginning with the one in greatest quantity were pentadecane, tridecane, and undecane (Table 2). The substances found at highest retention times (peak numbers 84, 93, 94, 96–99, and 105 in Table 2) corresponded to long-chain esters. Some samples from locations t1 10b and t1 10c also contained some linear and branched ketones, mainly 2-pentadecanone and 2-heptadecanone, which in other samples were present in tiny amounts or were undetectable.

C. savignyi, Egypt

In general, the Dufour gland contents of the colonies collected in Egypt were similar to those of the same species samples collected in Tunisia. For example,

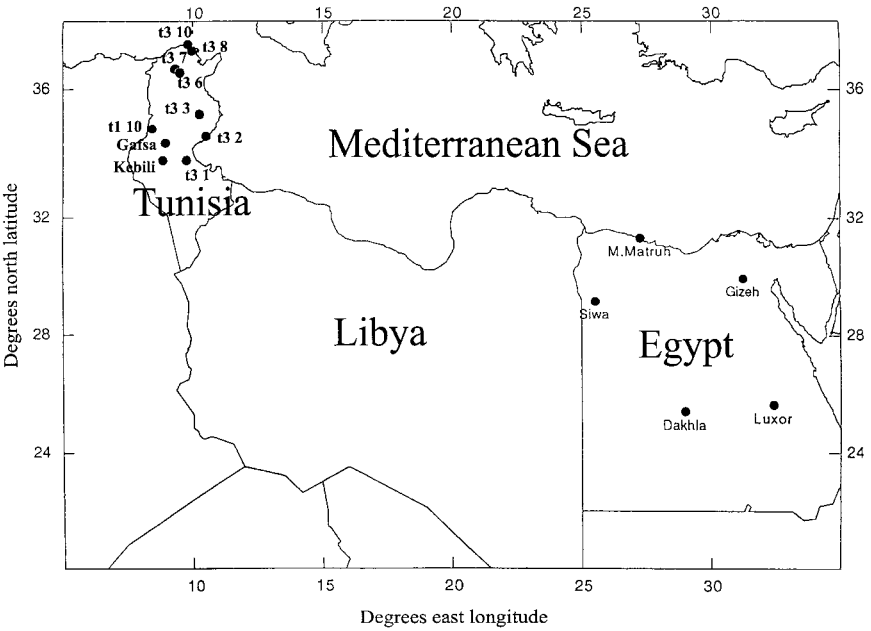


FIG. 1. The collection sites of nests of *Cataglyphis* ants in Tunisia and Egypt. The locations in Tunisia are explained in the text.

in all the Egyptian specimens, the order of the major compounds was pentadecane, tridecane, and undecane as in the Tunisian samples. However, undecane and tridecane were present in a significantly higher amount in the Tunisian samples (t test on the transformed data, respectively $t = 4.11$, $P < 0.001$ and $t = 4.44$, $P < 0.001$), while there was proportionally more of tetradecane and heptadecane in the Egyptian samples ($t = 7.55$, $P < 0.001$ and $t = 2.01$, $P = 0.048$, respectively). Moreover, in the Egyptian samples, the average percentage of branched hydrocarbons is almost twice the value found in the Tunisian specimens (13.5 and 7.4, respectively; t test on the transformed data $t = 4$, $P < 0.001$). No relevant differences were noted between the two samples for the non-hydrocarbon components.

C. bicolor (bdb)

The immediate difference noted in the bdb samples, collected across transect 3 in Tunisia, was their higher proportion of esters compared with the *savignyi* samples. All the esters identified were also found in the *savignyi* samples (Table 2), with the exception of those corresponding to peak numbers 85, 86, 102, and 103.

TABLE 2. AVERAGE PERCENTAGES ± STANDARD DEVIATION OF THE COMPOUNDS FOUND IN THE SIX *Cataglyphis* SPECIES ANALYZED*

Peak number	Compound name	*C. savignyi* Tunisia (bdw)	*C. savignyi* Egypt	*C. bicolor* Tunisia (bdb)	*C. viaticus* Tunisia (bb)	*C.diehlii* Tunisia	*C. bombycinus* Tunisia
1	Undecene	*t*	*t*	—	—	—	—
2	Undecane	**11.7 ± 6.5**	**6.6 ± 2.3**	**11.9 ± 4.7**	**5.6 ± 2.1**	3.7 ± 3.1	0.7 ± 0.4
3	5-Methylundecane	0.2 ± 0.2	0.6 ± 0.3	*0.1 ± 0.1*	*0.1 ± 0.1*	—	
4	3-Methylundecane	1.7 ± 1.0	1.4 ± 0.7	*0.1 ± 0.1*	0.4 ± 0.3	—	
5	Dodecane	1.2 ± 0.6	1.5 ± 0.3	1.5 ± 0.3	2.1 ± 0.4	—	0.2 ± 0.1
6	3-Methyldodecane	—	0.1 ± 0.1	—	0.2 ± 0.1	—	
7	Tridecene	*0.3 ± 0.4*[1]	*0.2 ± 0.1*[1]	0.1 ± 0.1	0.3 ± 0.0	0.1 ± 0.1	0.1 ± 0.0
8	Tridecane	**23.1 ± 6.4**	**17.1 ± 4.0**	**22.5 ± 6.2**	**32.7 ± 4.5**	**45.1 ± 23.5**	**13 ± 0.4**
9	7-Methylridecane	—	*0.1 ± 0.2*	—	*t*	—	—
10	5-Methyltridecane	0.6 ± 0.2	*0.7 ± 0.7*	—	0.5 ± 0.2	—	*t*
11	3-Methyltridecane	2.0 ± 0.6	3.2 ± 1.5	1.5 ± 0.4	**7.0 ± 1.1**	0.1 ± 0.1	0.8 ± 0.4
12	Tetradecene	*0.1 ± 0.1*	0.3 ± 0.2	0.2 ± 0.1	0.3 ± 0.2[1]	—	—
13	Tetradecane	3.1 ± 0.7	**5.3 ± 1.1**	3.5 ± 0.5	2.7 ± 0.6	0.1 ± 0.1	2.3 ± 0.4
14	3,11-Dimethyltridecane	—	*0.1 ± 0.1*	—	0.2 ± 0.1	—	—
15	5-Methyltetradecane	*t*	*0.1 ± 0.1*	—	—	—	—
16	4-Methyltetradecane	—	*0.1 ± 0.1*	—	0.2 ± 0.1	—	—
17	3-Methyltetradecane	—	*0.1 ± 0.1*	—	*0.1 ± 0.1*	—	*0.1 ± 0.1*
18	Pentadecadiene	—	—	—	*0.1 ± 0.1*	—	—
19	Pentadecene	0.8 ± 0.8[1]	0.4 ± 0.4	0.7 ± 0.8[1]	2.1 ± 1.7[1]	**17.6 ± 12.7**	0.5 ± 0.4
20	Pentadecane	**38.7 ± 11.7**	**37.1 ± 6.6**	**36.0 ± 7.3**	**20.2 ± 3.3**	**7.9 ± 7.0**	**44.3 ± 0.4**
21	2-Tridecanol	—	—	—	*0.1 ± 0.1*	—	—
22	4,11-Dimethyltetradecane	—	—	—	0.5 ± 0.3	—	—
23	Unidentified	—	—	—	*t*	—	—
24	7-Methylpentadecane	0.6 ± 0.5	*0.8 ± 0.8*	*t*	*0.1 ± 0.1*	—	*0.1 ± 0.1*
25	5-Methylpentadecane	1.5 ± 0.7	2.9 ± 1.6	1.7 ± 1.1	2.3 ± 1.3	—	2.1 ± 0.7

TABLE 2. CONTINUED

Peak number	Compound name	*C. savignyi* Tunisia (bdw)	*C. savignyi* Egypt	*C. bicolor* Tunisia (bdb)	*C. viaticus* Tunisia (bb)	*C.diehlii* Tunisia	*C. bombycinus* Tunisia
26	3-Methyl-2-tridecanone	—	—	—	—	—	0.2 ± 0.1
27	3-Methylpentadecane	0.7 ± 0.4	3.3 ± 2.0	*0.4 ± 0.3*	1.3 ± 0.6	—	4.5 ± 2.3
28	Hexadecene	0.1 ± 0.2	0.3 ± 0.3	0.1 ± 0.1[1]	0.2 ± 0.1	0.5 ± 1.0	0.2 ± 0.1
29	Hexadecane	*0.8 ± 0.6*	0.9 ± 0.9	0.5 ± 0.4	*0.4 ± 0.2*	0.2 ± 0.2	2.4 ± 0.9
30	3-Tetradecanone	—	—	—	*0.1 ± 0.2*	—	—
31	2-Tetradecanone	*t*	*0.1 ± 0.1*	*t*	0.3 ± 0.1	—	0.2 ± 0.1
32	2-Tetradecanol	—	*t*	*t*	*t*	—	—
33	Dodecyl acetate	—	—	*t*	*0.2 ± 0.2*	**15.0 ± 12.8**	1.7 ± 0.5
34	3-Methyldodecyl acetate	—	—	—	—	0.2 ± 0.3	—
35	7-Methylhexadecane	—	*t*	—	—	—	—
36	5- Methylhexadecane	—	*t*	—	—	—	—
37	3-Methylhexadecane	—	*t*	—	—	—	—
38	x-Methyl-2-tetradecanone	—	*t*	—	—	—	—
39	3-Methyl-2-tetradecanone	—	—	—	—	—	*0.1 ± 0.1*
40	2-Tridecyl acetate	—	—	—	*0.2 ± 0.2*	—	—
41	10-Methyldodecyl acetate	—	—	—	*0.4 ± 0.5*	—	—
42	Heptadecadiene	*t*	*0.1 ± 0.1*	—	—	—	0.5 ± 0.2
43	2-Methyltetradecanal	*t*	*0.2 ± 0.2*	*0.3 ± 0.4*	0.7 ± 0.2	—	—
44	Heptadecene	*0.2 ± 0.3*	*t*	*t*	*0.2 ± 0.3*	2.1 ± 2.4	0.9 ± 0.3
45	Heptadecane	**5.4 ± 4.8**	**7.6 ± 3.4**	1.6 ± 1.5	0.4 ± 0.1	1.4 ± 0.9	**15.8 ± 1.0**
46	Dodecyl propionate	—	—	—	—	—	*0.6 ± 0.8*
47	2-Pentadecanone	*0.4 ± 0.4*	*0.4 ± 0.7*	*0.2 ± 0.2*	1.0 ± 0.5	—	*4.1 ± 5.7*
48	Tridecyl acetate	—	—	—	*t*	1.0 ± 1.0	*0.1 ± 0.1*
49	7-Methylheptadecane	*t*	*0.2 ± 0.2*	—	—	—	*0.1 ± 0.1*
50	5-Methylheptadecane	*t*	*0.2 ± 0.2*	—	—	—	0.2 ± 0.1

51	Unidentified	—	—	*t*	—	—	—
52	Dodecyl isobutyrate	—	—	—	—	—	*0.4 ± 0.5*
53	2-Methylpentadecanal	—	—	—	*0.1 ± 0.1*	—	—
54	3-Methyl-2-pentadecanone	*t*	*t*	—	—	—	1.0 ± 0.2
55	3-Methylheptadecane	*t*	*0.1 ± 0.1*	—	—	—	0.4 ± 0.2
56	x-Methyl-2-pentadecanone	*t*	*t*	—	*0.1 ± 0.1*	—	*0.4 ± 0.5*
57	Octadecane	—	*0.2 ± 0.2*	—	—	—	*0.1 ± 0.1*
58	3-Hexadecanone	*t*	*0.1 ± 0.1*	—	0.3 ± 0.2	—	0.4 ± 0.3
59	2-Hexadecanone	*t*	*0.1 ± 0.1*	*t*	—	—	*0.1 ± 0.1*
60	2-Hexadecanol	—	*t*	—	—	—	—
61	Dodecyl butyrate	—	—	—	—	—	0.2 ± 0.2
62	Tridecyl propionate	—	—	—	—	—	*0.1 ± 0.1*
63	Tetradecyl acetate	—	—	—	*0.1 ± 0.3*	2.6 ± 3.5	0.4 ± 0.3
64	Tridecyl isobutyrate	—	—	—	—	—	*0.1 ± 0.1*
65	12-Methyltetradecyl acetate	—	—	—	*0.2 ± 0.2*	—	—
66	Nonadecadiene	—	*t*	—	—	—	—
67	Nonadecene	*0.1 ± 0.2*	*0.1 ± 0.1*	—	—	—	—
68	2-Methylhexadecanal	*t*	*0.1 ± 0.1*	*0.1 ± 0.1*	*0.1 ± 0.1*	—	—
69	Nonadecane	*0.4 ± 0.4*	*1.7 ± 1.7*	*0.1 ± 0.1*	—	—	0.2 ± 0.1
70	Tetradecyl propionate	—	—	—	—	—	*0.1 ± 0.1*
71	2-Heptadecanone	*0.7 ± 1.2*	*1.9 ± 1.5*	*0.1 ± 0.2*	—	—	*0.4 ± 0.5*
72	Pentadecyl acetate	—	—	—	—	—	0.2 ± 0.2
73	Tetradecyl isobutyrate	—	—	—	—	—	*0.1 ± 0.1*
74	Hexadecyl acetate	—	—	—	—	—	*0.1 ± 0.1*
75	3-Methylnonadecane	—	*t*	—	—	—	—
76	x-Methyl-2-heptadecanone	*t*	*0.1 ± 0.1*	—	—	—	—
77	3-Octadecanone	—	*t*	—	—	—	—
78	2-Octadecanone	—	*t*	—	—	—	—
79	2-Methylhexyl hexadecanoate	—	—	—	—	0.1 ± 0.2[3]	—
80	2-Methylhexyl 12-methylhexadecanoate	—	—	—	—	0.1 ± 0.4[3]	—

TABLE 2. CONTINUED

Peak number	Compound name	*C. savignyi* Tunisia (bdw)	*C. savignyi* Egypt	*C. bicolor* Tunisia (bdb)	*C. viaticus* Tunisia (bb)	*C.diehlii* Tunisia	*C. bombycinus* Tunisia
81	2-Methylhexyl 10-methylhexadecanoate	—	—	—	—	0.1 ± 0.2[3]	—
82	2-Methylhexyl heptadecanoate	—	—	—	—	0.1 ± 0.2[3]	—
83	2-Methylhexyl 13-methylheptadecanoate	—	—	—	—	0.1 ± 0.2[3]	—
84	Dodecyl decanoate	*0.1 ± 0.1*	—	2.0 ± 0.7	1.0 ± 0.8	—	—
85	Mixture of isomeric C_{23} esters	—	—	0.4 ± 0.2	—	—	—
86	Mixture of dodecyl undecanoate & undecyl dodecanoate	—	—	*0.2 ± 0.1*	—	—	—
87	2-Methylhexyl octadecenoate	—	—	—	—	0.2 ± 0.6[3]	—
88	2-Methylhexyl octadecanoate	—	—	—	—	0.1 ± 0.2[3]	—
89	Dodecyl x-methyldecanoate	*t*	—	—	2.6 ± 1.3	—	—
90	Mixture of branched C_{23} esters	—	—	—	1.1 ± 0.7	—	—
91	Mixture of branched undecyl dodecanoate & branched dodecyl undecanoate	—	—	—	0.5 ± 0.4	—	—
92	Mixture of branched C_{24} esters	—	—	—	0.5 ± 0.4	—	—
93	Dodecyl dodecenoate	*0.1 ± 0.2*	—	—	—	—	—
94	Dodecyl dodecanoate	*0.5 ± 0.5*	*0.2 ± 0.4*	4.1 ± 1.5	2.3 ±1.4	0.2 ± 0.5	—
95	13-Methylheptacosane	—	—	—	—	0.2 ± 0.2	—

96	Dodecyl x-methyldodecanoate	*0.4 ± 0.3*	*0.1 ± 0.1*	0.7 ± 0.3	2.0 ± 0.9	—	—
97	Dodecyl 6-methyldodecanoate	*0.2 ± 0.2*	*t*	0.8 ± 0.3	*0.9 ± 0.7*	—	—
98	Dodecyl 10-methyldodecanoate	*0.3 ± 0.3*	*0.1 ± 0.1*	1.3 ± 0.6	*1.8 ± 1.4*	—	—
99	Mixture of branched dodecyl tridecanoate & branched tridecyl dodecanoate	*0.1 ± 0.2*	—	—	—	—	—
100	Mixture of branched C_{25} and C_{26} esters	—	—	—	*1.6 ± 1.2*	—	—
101	Mixture of C_{25} esters	—	*0.1 ± 0.1*	—	—	—	—
102	Mixture of dodecyl tridecanoate & tridecyl dodecanoate	—	—	*0.3 ± 0.3*	—	—	—
103	Mixture of isomeric C_{25} esters	—	—	*0.1 ± 0.2*	—	—	—
104	Dodecyl tetradecenoate	*0.3 ± 0.5*	—	0.1 ± 0.2		—	—
105	Dodecyl tetradecanoate	*1.5 ± 1.7*	*1.2 ± 1.7*	5.4 ± 2.2	1.0 ± 0.4	0.3 ± 0.6	—
106	Mixture of C_{26} esters	—	*0.2 ± 0.4*	—		—	—
107	Mixture of branched C_{26} and C_{27} esters	—	—	—	*0.3 ± 0.6*	—	—
108	Mixture of branched dodecyl pentadecanoate & branched tridecyl tetradecanoate	*0.6 ± 0.6*	*0.3 ± 0.4*	—	0.3 ± 0.1	—	—

TABLE 2. CONTINUED

Peak number	Compound name	*C. savignyi* Tunisia (bdw)	*C. savignyi* Egypt	*C. bicolor* Tunisia (bdb)	*C. viaticus* Tunisia (bb)	*C.diehlii* Tunisia	*C. bombycinus* Tunisia
109	Dodecyl pentadecanoate	*0.1 ± 0.2*	*0.2 ± 0.3*	0.4 ± 0.3[2]	*0.1 ± 0.1*	—	—
110	Mixture of C_{27} esters	—	*t*	—	—	—	—
111	Dodecyl hexadecanoate	*0.4 ± 0.6*	1.0 ± 1.0	*0.9 ± 0.7*	—	0.8 ± 1.6	—
112	Dodecyl heptadecanoate	—	*0.3 ± 0.3*	—	—	—	—

*Average values higher than 5.0% are written **bold**; when value is written in *Italic* the compound was not found in all the colonies (this does not apply to *C. diehlii* for which only one colony was available); when the value is underlined not all the individuals belonging to the same colony contained the compound in detectable amounts.
[1]Present as two isomers in some specimens.
[2]The peak also contains tridecyl tetradecanoate.
[3]Only present in one of the 9 specimens analysed (the same specimen for all the compounds indicated with this number.

However, some of the esters were present in much higher proportion, as dodecyl tetradecanoate (peak 104, 5.4%), dodecyl dodecanoate (peak 94, 4.1%), dodecyl decanoate (peak 84, 2.0%) and dodecyl 10-methyldodecanoate (peak 98, 1.3%) ($8.02 \leq t \leq 13.78$, $P < 0.001$). These esters were almost constantly present among all the samples analyzed (only one specimen out of 47 lacked both dodecyl dodecanoate and dodecyl tetradecanoate, one lacked decyl decanoate, and four lacked dodecyl 10-methyldodecanoate). Among the colonies, the average total proportion of esters varied from 4% to 22%. Despite this variation, the total percentage of esters in *C. bicolor* was higher than in the Tunisian *C. savignyi* (averages respectively, 16.08% and 4.68%; *t* test on the transformed data $t = 8.39$, $P < 0.001$).

With regard to the hydrocarbons, the average values were close among the Tunisian *savignyi* and the *bicolor* samples, with the exception of heptadecane which was in a lower amount in *bicolor* ($t = 5.70$, $P < 0.001$). By comparing the sympatric bdw and bdb samples collected on location 2 on transect 3, the same differences as reported above were noted.

C. viaticus (bb)

Samples given the code name bb contained the same hydrocarbons and esters as found in the other groups already described, but the percentage values were obviously different from the others (Table 2). First, all but 3 samples contained tridecane as the major component and pentadecane as second. Secondly, they all had a branched alkane, 3-methyltridecane, as an important component (*t* test for the above compounds against Tunisian *savignyi* and *bicolor*: $3.5 \leq t \leq 17.3$, $0.001 \leq P < 0.001$). Moreover, undecane and heptadecane were in lower amounts than in the Tunisian *savignyi* and in the *bicolor* samples ($5.30 \leq t \leq 9.6$; $P < 0.001$).

The amount of esters ranged among the colonies from 10 to 24%. However, compared to the *bicolor* samples, instead of dodecyl esters of tetradecanoic, dodecanoic, and decanoic acids dominating the esters (all in a significantly lower amount in the *viaticus* samples $3.57 \leq t \leq 9.93$, $P < 0.001$), there was a more complex mixture including more branched esters. Thus, the pattern of esters in the chromatogram looked quite different. The branched esters corresponding to the peak numbers 90–92 and 100, which all together gave 3.7% of the total, were only found in this species, while dodecyl x-methyldecanoate, corresponding to peak 89, whose average amount was 2.6%, was present only in traces in the Tunisian *savignyi* and in the *bicolor* samples. All these compounds were present in all the *viaticus* colonies analyzed. Four acetates were found (peak numbers 40, 41, 63, and 65), which were not found in the other samples, but their presence was variable both among colonies and among individuals belonging to the same colony (Table 2).

C. diehlii

One group of specimens, all belonging to the same colony, labelled diehlii from Methaoui in Tunisia was different from the others containing a simpler mixture of substances (Table 2). There was low consistency of composition in these nine specimens, as reflected in the larger standard deviation in Table 2, compared to all the other samples. As in the bb samples, the major component was tridecane ($t = 1.70$, n.s. in the comparison with the bb samples; $2.86 \leq t \leq 3.79$, $0.021 \leq P \leq 0.005$ in the comparison with bdb, bdw and Egyptian *savignyi*), but the amount of pentadecane and undecane was lower than in all the other samples analysed (respectively, $3.48 \leq t \leq 7.44$, $0.004 \leq P < 0.001$; $1.97 \leq t \leq 5.56$, $0.073 \leq P < 0.001$ in comparison with bdw, Egyptian *savignyi*, bdb and bb). Moreover a higher percentage of pentadecene was present (comparison with bb samples, $t = 2.96$, $P = 0.018$), despite the amount being variable among specimens. The unresolved peak of pentadecene and pentadecane was integrated by plotting single ion chromatograms for m/z values 210 and 212, respectively. Dodecyl acetate, only found in traces in the other samples, was present in a high average amount (15%), although it was absent in 2 out of the 9 analyzed glands. Tetradecyl acetate was also present in a high average amount (2.6%), but was missing in four out of the nine samples.

It is noteworthy that we found esters with a common ion at m/z 98 in their mass spectra in one specimen of this group. We determined these to be 2-methylhexyl esters of fatty acids. The esters with branched-chain acid portions gave clear strong ions at the branching points. For example, 2-methylhexyl 12-methylhexadecanoate had a weak molecular ion (M^+ 368, intensity 2%), and ions at m/z 311 (M-C_4H_7, 2%), 271 ($RCO_2H_2^+$, 20%), 270 (RCO_2H, 16%), 253 (RCO^+, 18%), 213 ($RCO_2H-C_4H_7$, 19%), 98 ($C_7H_{14}^+$ from the 2-methylhexanol, 53%) and 57 (C_4H_7, from both acid and alcohol parts, base peak). In 2-methylhexyl 13-methylheptadecanoate, the lower mass ions were unchanged and the high mass ions were shifted to 382 (M^+), 325, 285, 284, 267, and 227, respectively. Since all members of the *bicolor* group have the necessary enzymes to produce 2-methylhexanol in their mandibular glands, it is interesting to see this substance bound as esters in their Dufour glands. If similar esters are present in the other *bicolor* species, they are below our limit of detection.

C. bombycinus

The absence of 2-methyl-1-hexanol and the presence of citronellol and citral in the mandibular glands of the samples Gafsa bom and Kebili bom drew attention to these samples as not belonging to the group we had come to recognize as *bicolor*. As for most of the *bicolor* group, the major hydrocarbon was pentadecane (Table 2), but its average percentage was higher than in all the other samples analyzed

($2.22 \leq t \leq 11.82$, $0.032 \leq P < 0.001$). Tridecane was in a lower percentage amount than in all the other samples ($3.27 \leq t \leq 8.85$, $0.002 \leq P < 0.001$), and undecane was present in a low amount. Heptadecane was in a higher proportion than in all the *bicolor* samples ($7.43 \leq t \leq 32.18$, $P < 0.001$). Dodecyl acetate was found in about half of the samples, sometimes in high amounts; however, its proportion was lower than in the *diehlii* specimens ($t = 2.96$, $P = 0.016$). All together, the low standard deviation for the *bombycinus* indicated their homogeneity (Table 2), but the Gafsa sample lacked the traces of propionate esters present in the Kebili colony. The absence of further branching in peaks 26, 39, and 54, 3-methyl-2-tridecanone, 3-methyl-2-tetradecanone, and 3-methyl-2-pentadecanone (Table 2) cannot be excluded with as great a certainty as for the branched esters of *C. diehlii* above, and synthetic samples were not available. *C. bombycinus* is placed in the *bombycinus* group (Wehner et al., 1994).

DISCUSSION

Chemotaxonomy, or the use of chemical composition of an animal or plant, together with other criteria, to define a species has been in use for considerable time in particular cases. From a number of studies made of the secretion of the Dufour glands of ants (c.f. Morgan, 1992), it would appear that this secretion is relatively constant in composition and unique to each species. Because of the difficulty in diagnosing species in the large *Cataglyphis* genus, a chemotaxonomic study of exocrine secretions was undertaken (Keegans et al., 1992; Agosti et al., 1996). The group of large desert-dwelling *Cataglyphis* ants of Tunisia has been refined into five species (Wehner et al., 1994), four in the *bicolor* group (with 2-methyl-1-hexanol as their characteristic mandibular gland substance) and one in the *bombycinus* group (with citronellol and citral in the mandibular glands). Among the *bicolor* group species, *C. savignyi*, *C. bicolor*, and *C. viaticus* were quite similar for the composition of the Dufour's gland secretion, while *C. diehlii* was markedly differed from these three species showing (i) a much less complicated mixture and (ii) a high concentration of dodecyl acetate, a compound only found in low concentrations in some specimens of *C. viaticus* and in *C. bicolor*, and (iii) the presence of the 2-methylhexyl esters of long chain fatty acids, unique among the five species analyzed. Thus, the Dufour gland chemistry appears to be a clear diagnostic character for *C. diehlii*, while based on the postpharyngeal gland secretion, *C. diehlii* is not readily distinguishable from *C. bicolor*. Although less evident, differences between C. *savignyi*, *C. bicolor*, and *C. viaticus* were found in the concentration of the major constituents. As might be expected for insects so geographically separated, the glands of *C. savignyi* from Egypt and Tunisia were chemically differentiated, and since this difference is of an extent comparable to that observed between species, the chemistry of the Dufour gland does not appear

to be a reliable character for species identification if based on the description of the composition of allopatric populations. We have found for these closely related species the contents of the postpharyngeal gland (Oldham et al., 1999) to be a better separator than the Dufour gland contents.

In contrast to the differences in the mandibular glands between the *bicolor* group and *C. bombycinus*, the Dufour gland chemistry did not differ remarkably between them. However, the two groups were differentiated by the lack of the high molecular weight constituents, mainly long chain fatty acid esters, in *C. bombycinus*. It can be seen from Table 2 that in all the species, several of the minor components were absent in some of the analyzed specimens and sometimes in all the specimens sampled from the same colony. Although this sort of variability is generally not discussed in papers describing the composition of exocrine volatile secretion in insects, we know from direct experience it is a common situation. Moreover, even considerable deviations from the average values of concentration for several of the compounds are common. In this sense, we believe that the composition of just one volatile exocrine secretion is rarely a reliable and readily usable taxonomic character, but may prove to be useful for species determination if considered together with other chemotaxonomic and anatomical characters.

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MAMMALIAN EXOCRINE SECRETIONS. XVII: CHEMICAL CHARACTERIZATION OF PREORBITAL SECRETION OF MALE SUNI, *Neotragus moschatus*

M. A. STANDER, B. V. BURGER,* and M. LE ROUX

Laboratory for Ecological Chemistry
Department of Chemistry
University of Stellenbosch
Stellenbosch 7600, South Africa

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Abstract—Gas chromatographic and gas chromatographic–mass spectrometric techniques were employed to identify 83 compounds, including alkanes, alkenes, aldehydes, 2-methylalkanes, carboxylic acids, 1-alkyl formates and alken-1-yl formates, benzoic acid, and cholesterol, in the preorbital secretion of the male suni, *Neotragus moschatus*. Dimethyl disulfide derivatization and lithium aluminum hydride reduction were used to determine the position of double bonds and to confirm the identity of the functional groups in some of the constituents of the secretion.

Key Words—Suni, *Neotragus moschatus*, semiochemicals, pheromones.

INTRODUCTION

Extensive research has been done on the role of the secretions of the preorbital glands of hoofed mammals or ungulates of the order Artiodactyla in their rutting and territorial behavior (e.g., Langguth and Jackson, 1980; Mossing and Damber, 1981; Frey and Hofmann, 1997; Marmazinskaya, 1997; Roberts, 1997, 1998; Arcese, 1999; Brashares and Arcese, 1999; Lawson et al., 2000, 2001), but in spite of the biological importance attributed to them, little is known about the chemical composition of preorbital secretions. Andersson (1979) has identified two ketones in the preorbital pouch secretion of the reindeer, *Rangifer t. tarandus*. Several heavy constituents such as cholesterol, lanosterol, fatty acids, and triglycerides were subsequently identified in this secretion (Sokolov et al., 1977). Cholesterol, benzaldehyde, and a homologous series of saturated γ-lactones have been identified

*To whom correspondence should be addressed. E-mail: lecus@maties.sun.ac.za

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in the preorbital secretion of the muskox, *Ovibos moschatus* (Flood et al., 1989). The preorbital secretions of the bontebok, *Damaliscus dorcas dorcas*, and blesbok, *D. d. phillipsi*, contain, among other compounds, some unbranched primary alcohols, a few short-chain saturated and several long-chain saturated and unsaturated unbranched carboxylic acids and aldehydes, three long-chain δ-lactones, benzoic acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, 2-heptanone, piperitone, dimethylsulfone, isopropyl tetradecanoate, isopropyl hexadecanoate, hexadecyl acetate, cholesterol, α-tocopherol, and squalene (Burger et al., 1999a).

With the exception of the female oribi, *Ourebia ourebi*, which does not produce a preorbital secretion (Mo et al., 1995), and the preorbital secretion of female klipspringer, *Oreotragus oreotragus*, which has not yet been investigated, the males and females of the other members of the subfamily Antilopinae produce qualitatively identical preorbital secretions. In sharp contrast, exploratory gas chromatographic–mass spectrometric (GC-MS) analyses of the preorbital secretions of suni, *Neotragus moschatus*, a dwarf antelope of the tribe Neotragini, revealed prominent qualitative and quantitative differences in the secretions of male and female animals. The suni was, therefore, selected for further study of its preorbital secretions in a continuing investigation of the role of preorbital secretions in the territorial behavior of South African antelope species.

METHODS AND MATERIALS

General. All Pyrex glassware was thoroughly cleaned with water and organic solvents and then heated at 500°C in an annealing oven to remove any trace of organic contaminants. Syringes and other apparatus that could not be heated to 500°C were cleaned with dichloromethane (Merck, Residue Analysis Grade). Preorbital secretions were extracted and the extracts diluted, where necessary, with this solvent.

Collection and Sample Preparation. Preorbital gland secretions were collected with a tubular PTFE scoop from male suni kept in pens (10 × 10 m) at the Tygerberg Zoo, Cape Town, South Africa. The scoop, with an inside diameter of 5 mm, was furnished with a PTFE plunger with which the collected material could be ejected into a 1-ml Reacti-Vial. Samples were taken at different times of the year as needed from six males bred from two unrelated lineages. To avoid the risk of losing animals, males were not trapped in the presence of pregnant females.

The organic constituents were extracted from the mucoid secretion by stirring the collected material with an appropriate quantity of dichloromethane, using a thin (ca.1 mm diam.) glass rod, centrifuging the resulting suspension at 3500 rpm for 15 min, and removing the organic fraction from underneath the supernatant mucoid layer with a 100-μl syringe. In a typical sample preparation, 108 mg of secretion was collected from the glands of a male and extracted with 120 μl of

dichloromethane to give 97 μl of extract. This extract was used without further concentration for quantitative determination of the volatile organic constituents. However, to avoid having to concentrate extracts for GC-MS analysis with the concomitant loss of volatiles, the organic material was mostly extracted with the smallest possible volume of solvent that still gave a separable solvent layer. Extracts were transferred to clean Reacti-Vials and stored at -30°C until used for analysis.

Analytical Methods. Instrumentation for the identification of the volatiles has been described in detail by Burger et al. (1996). GC and GC-MS analyses were done with the following capillary columns: (1) 40-m $\times$ 0.3-mm glass column coated with PS-089 (polarity equivalent to that of SE-52) at a film thickness of 0.25 μm, and (2) 30-m $\times$ 0.25-mm glass column coated with OV-240 at a film thickness of 0.25 μm. Helium was employed as carrier gas at a linear velocity of 28.6 cm/sec at 40°C. Samples were injected at an injector temperature of 220°C, thermally focused on the column at 30°C and analyzed using a temperature program of 2°C/min from 40°C to 270°C and holding the temperature at 270°C for 80 min. The flame ionization detector was operated at 280°C. Quantitative GC analyses were done with the same instrument and the PS-089 column, and data acquisition with Borwin Intuitive Chromatography Software (JMBS Developments, 38600 Fontaine, France) using hexadecanoic acid as external standard.

Low-resolution electron impact mass spectra (EI-MS) were obtained at 70 eV on a Carlo Erba QMD 1000 GC-MS instrument by using the columns and temperature program specified above. An ion source temperature of 100°C was used. Chemical ionization mass spectra, with methane as reactant gas [CI(CH_4)-MS], were obtained on an AMD 604 double-focusing mass spectrometer at a resolution of 1000 and a mass range of 100–500 atomic mass units.

Dimethyl Disulfide Derivatization. DMDS derivatization was carried out according to the method of Vincenti et al. (1987). A dichloromethane extract (18 μl) of the secretion was concentrated in a 1-ml Reacti-Vial with a slow stream of purified (activated charcoal) nitrogen. Residual dichloromethane was removed by the addition and slow evaporation of carbon disulfide 50 μl. This process was repeated three times. The residual material was dissolved in 50 μl carbon disulfide, and 5 μl of an iodine solution (60 mg iodine in 1 ml diethylether) as well as 50 μl dimethyl disulfide were added. The screw-capped vial was sealed using a PTFE-lined septum and left in the oven of a gas chromatograph at 60°C for 40 hr. The reaction was quenched with an aqueous solution of sodium thiosulfate (5%), and the DMDS derivatives were isolated by centrifuging the reaction mixture for a few minutes at 2000 rpm. The organic layer was transferred to a clean Reacti-Vial with a 100-μl syringe and concentrated to 5 μl for GC-MS analysis.

Lithium Aluminium Hydride Reduction. The organic constituents of the secretion were subjected to lithium aluminum hydride reduction to confirm the presence of reducible functional groups. A 7-μl aliquot of the extract was evaporated to dryness in a stream of nitrogen. The residual material was redissolved

twice in diethyl ether, evaporated to eliminate traces of dichloromethane, and then dissolved in diethyl ether (30 μl) and treated with a saturated solution of $LiAlH_4$ in ether (20 μl). The Reacti-Vial was closed using a PTFE-lined septum and heated for 5 min at 40°C, after which the reaction mixture was cooled and treated with cold water (30 μl), centrifuged, the organic layer transferred to a clean Reacti-Vial, and the resulting solution concentrated for GC-MS analysis.

Reference Compounds. Some of the compounds identified in the preorbital secretion of the male suni were available from previous research projects in this series, while others were obtained commercially. Unsaturated alcohols were purchased from Pherobank (Wageningen, The Netherlands). The following compounds were synthesized.

2-Methylicosane. A mixture of 1-bromohexadecane (2.78 g, 0.01 mol), 1-bromo-3-methylbutane (1.51 g, 0.01 mol), and sodium (0.92 g, 0.04 mol) was refluxed under argon until the Wurtz condensation started. The reaction went to completion within 3 hr. The unreacted sodium was destroyed with ethanol, whereafter water (5 ml) was added to the reaction mixture and the organic material extracted with hexane. According to GC-MS analysis, the extract contained 2,7-dimethyloctane (26%), 1-hexadecene (49%), 1-hexadecanol (6%), 2-methylicosane (18%), and dotriacontane (1%). EI-MS of 2-methylicosane: m/z 43 (85), 57 (100), 71 (66), 85 (45), 99 (18), 113 (8), 127 (6), 141 (5), 155 (4), and 253 (4%).

1-Alkyl Formates. A mixture of the primary alcohols, 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, 1-nonanol, 1-decanol, 1-undecanol, 1-dodecanol, 1-tetradecanol, 1-hexadecanol, 1-octadecanol, 1-icosanol, 1-docosanol (15 mg each), and formic acid (420 μl) was heated overnight at 80°C and the products extracted with hexane for comparison with the constituents of the secretion.

Alken-1-yl Formates. A mixture of (*Z*)- and (*E*)-6-tridecen-1-yl formate, (*Z*)- and (*E*)-8-tetradecen-1-yl formate, and (*Z*)- and (*E*)-8-hexadecen-1-yl formate was prepared by heating the corresponding alkenols with formic acid and extracting the products from the reaction mixture as described above for the preparation of the saturated formates.

RESULTS AND DISCUSSION

The preorbital secretions of male and female suni differ both qualitatively and quantitatively, although they do contain a few constituents that are common to both. Male glands appear to be more productive than those of females, and the male secretion is also more complex. A series of related compounds forming the bulk of the female secretion could not be identified despite the availability of extensive 600-MHz NMR, HR-MS, and other physical data. Therefore, only the male secretion will be discussed here.

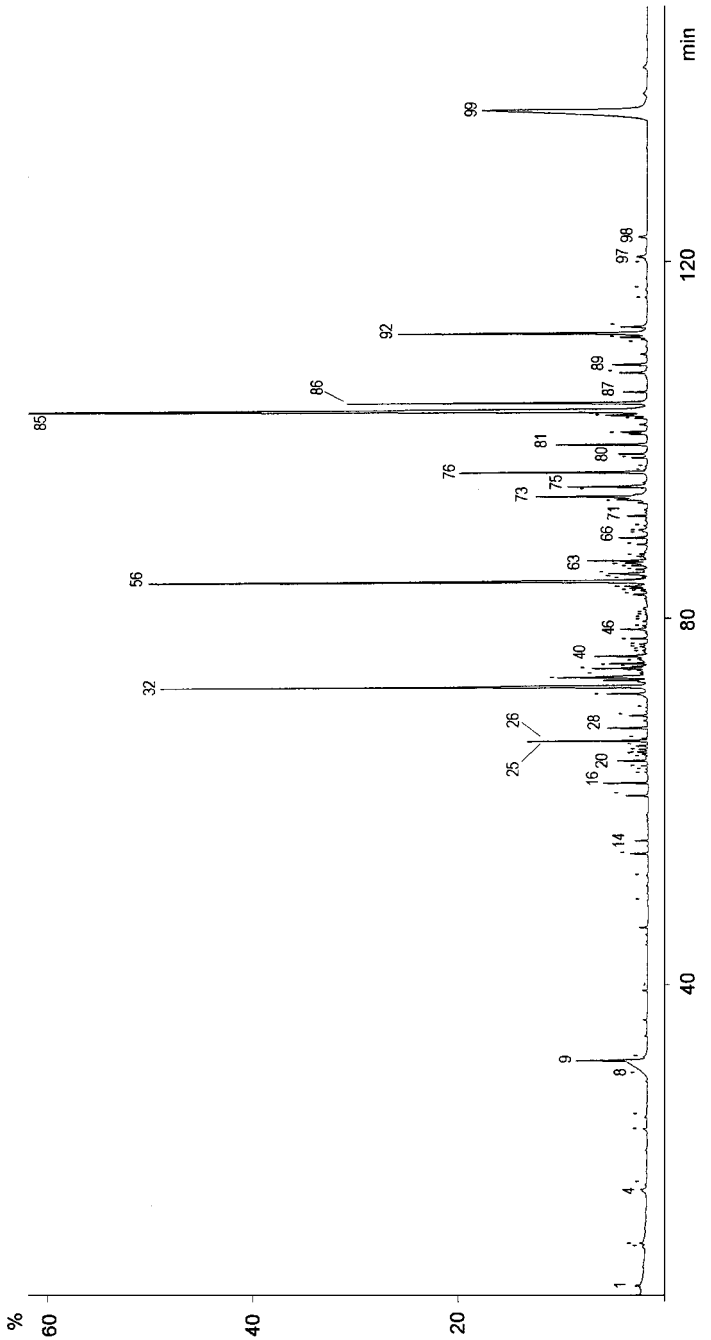


FIG. 1. Total ion chromatogram of an extract of the preorbital secretion of male suni, *Neotragus moschatus*. Glass capillary column coated with a 0.25-μm film of the apolar stationary phase PS-089, programmed at 2°C/min from 40°C to 270°C (hold).

A typical total ion chromatogram of the male secretion is shown in Figure 1. At the level of sensitivity at which the qualitative analyses were done, secretions from individual males were identical. Constituents from the male were tentatively identified by comparison of their mass spectra with mass spectral data in the NBS and Wiley mass spectral libraries. The identification of many compounds could be confirmed by coinjection of commercially available reference compounds or synthesized material. The molecular mass of some of the compounds and, therefore, their chain lengths were confirmed by CI(CH_4)-MS data.

There are two homologous series of alkanes present in the secretion. The first consists of long-chain, unbranched alkanes, the structures of which could easily be confirmed by GC coelution with reference compounds, and the second series contains saturated hydrocarbons with the typical EI mass spectra of isoalkanes. 2-Methylicosane was synthesized as a model compound to confirm interpretation of the mass spectral data. A series of unbranched, long-chain fatty acids, compounds that appear to be almost ubiquitous in the exocrine secretions of various antelope species, were also found in this secretion. These and the other compounds identified in the secretion are listed in Table 1.

Dimethyl disulfide derivatization and GC-MS analysis of the DMDS derivatives were used to determine the presence and position of double bonds in the unsaturated constituents of the secretion (Vincenti et al., 1987). The interpretation of the mass spectra of the DMDS derivatives was discussed in detail previously (Burger et al., 1996). Due to the complexity of the male secretion and extensive coelution of its constituents, almost none of the peaks in the chromatogram in Figure 1 represent pure compounds. With the exception of a few alkenyl formates identified using the PS-089 column, it was impossible to correlate the unsaturated constituents with their DMDS derivatives using the apolar PS-089 column. However, an OV-240 capillary column gave better separation of the large number of alkenes, saturated and unsaturated formic acid esters, and DMDS derivatives of the unsaturated compounds than the PS-089 column. The unsaturated compounds are listed in Table 1 in the order in which they eluted from the OV-240 column. Although these compounds do not necessarily elute in the same order from the less polar PS-089 column, the numbers are used in Figure 1 to indicate approximately where the different homologous groups of unsaturated compounds elute in the total ion chromatogram. In a few cases, DMDS derivatization showed both the *E* and *Z* isomers of the unsaturated alkenes and formates present in the secretion. It was, therefore, possible to assign *E* or *Z* configuration to the double-bond isomers based on the GC elution order of these compounds and their DMDS derivatives. The double-bond configuration of a few of the unsaturated constituents was also determined by GC retention time comparison with authentic samples. The elucidation of the stereochemistry of all unsaturated and chiral constituents of the secretion was not attempted. The stereochemistry of some of the unsaturated and

TABLE 1. COMPOUNDS IDENTIFIED IN PREORBITAL SECRETION OF MALE SUNI

No. in Fig.1 (PS-089 column)	Compound (arranged according to compound type)	Analytical method[a]	Quantity (μg/animal)
13	Pentadecane	a,b,d	46
25	Heptadecane	a,b,d	137
45	Nonadecane	a,b	45
65	Henicosane	a,b,d	23
79	Tricosane	a,b,d	23
12	2-Methyltetradecane	a,d	1
20	2-Methylhexadecane	a	76
30	2-Methylheptadecane	a,d	5
40	2-Methyloctadecane	a,d	169
63	2-Methylicosane	a,b,d	208
71	2-Methylhenicosane	a,d	59
76	2-Methyldocosane	a,d	300
82	2-Methyltricosane	a	48
87	2-Methyltetracosane	a,d	46
9	Impurity	a	
17	(*Z*)-6-Heptadecene	a,e,g	7
18	5-Heptadecene	a,e,g	6
19	(*Z*)-4-Heptadecene	a,e,g	15
21	7-Heptadecene	a,e,g	9
22	(*E*)-6-Heptadecene	a,e,g	16
23	(*E*)-4-Heptadecene	a,e,g	13
24	Unidentified heptadecene	a,h	21
35	(*Z*)-7-Nonadecene	a,e,g	16
36	6-Nonadecene	a,e,g	133
37	5-Nonadecene	a,e,g	31
38	9-Nonadecene	a,e,g	81
39	(*E*)-7-Nonadecene	a,e,g	11
41	4-Nonadecene	a,e,g	9
42	Unidentified nonadecene	a,h	5
43	Unidentified nonadecene	a,h	11
44	Unidentified nonadecene	a,h	14
48	5-Icosene	a,e,g	3
49	Unidentified icosene	a,h	3
50	Unidentified icosene	a,h	4
58	8-Henicosene	a,e,g	117
59	(*Z*)-9-Henicosene	a,e,g	103
60	10-Henicosene	a,e,g	31
61	7-Henicosene	a,e,g	68
62	6-Henicosene	a,e,g	141
64	(*E*)-9-Henicosene	a,e,g	23
68	9-Docosene	a,e,g	17
69	10-Docosene	a,e,g	14
74	9-Tricosene	a,e,g	3
77	10-Tricosene	a,e,g	5
78	11-Tricosene	a,e,g	5

TABLE 1. CONTINUED

No. in Fig.1 (PS-089 column)	Compound (arranged according to compound type)	Analytical method[a]	Quantity (μg/animal)
1	Hexanal	a,b,f	2
3	Heptanal	a,b,f	2
5	Octanal	a,b,f	0.5
7	Nonanal	a,b,f	1
2	Pentanoic acid	a,b,f	1
4	Hexanoic acid	a,b,f	3
6	Heptanoic acid	a,b,f	1
8	Benzoic acid	a,b,f	23
10	Octanoic acid	a,b,f	1
51	Hexadecanoic acid	a,b,f	7
70	Octadecanoic acid	a,b,f	26
11	1-Undecyl formate	a,b,c,d,f	5
14	1-Dodecyl formate	a,b,c,d,f	34
16	1-Tridecyl formate	a,b,c,d,f	107
28	1-Tetradecyl formate	a,b,c,d,f	103
34	1-Pentadecyl formate	a,f	73
47	1-Hexadecyl formate	a,b,c,f	3
57	1-Heptadecyl formate	a,d,f	25
67	1-Octadecyl formate	a,b,c,f	14
75	1-Nonadecyl formate	a,d,f	195
81	1-Icosyl formate	a,b,c,d,f	273
86	1-Henicosyl formate	a,d,f	762
89	1-Docosyl formate	a,b,c,d,f	162
93	1-Tricosyl formate	a,d,f	130
95	1-Tetracosyl formate	a,f	30
15	(*Z*)-6-Tridecen-1-yl formate	a,c,e,f,g	57
26	7-Tetradecen-1-yl formate	a,e,f,g	42
27	(*Z*)-8-Tetradecen-1-yl formate	a,c,e,f,g	19
32	8-Pentadecen-1-yl formate	a,d,e,f	782
53	8-Heptadecen-1-yl formate	a,e,f,g	14
54	10-Heptadecen-1-yl formate	a,d,e,f	4
72	10-Nonadecen-1-yl formate	a,e,f,g	60
73	12-Nonadecen-1-yl formate	a,d,e,f	171
80	13-Icosen-1-yl formate	a,e,f,g	107
83	12-Henicosen-1-yl formate	a,e,f,g	72
84	16-Henicosen-1-yl formate	a,e,f,g	206
85	14-Henicosen-1-yl formate	a,d,e,f	2319
88	15-Docosen-1-yl formate	a,e,f,g	142
90	14-Tricosen-1-yl formate	a,e,f,g	53
91	18-Tricosen-1-yl formate	a,e,f,g	137
92	16-Tricosen-1-yl formate	a,d,e,f	761
94	17-Tetracosen-1-yl formate	a,e,f,g	27
96	16-Pentacosen-1-yl formate	a,e,f,g	36
97	18-Pentacosen-1-yl formate	a,e,f,g	106
98	Unidentified steroid	a,b,f	2

TABLE 1. CONTINUED

No. in Fig.1 (PS-089 column)	Compound (arranged according to compound type)	Analytical method[a]	Quantity (μg/animal)
99	Cholesterol	a,b,f	1848
29	Unidentified		149
31	Unidentified		96
33	Unidentified		197
46	Unidentified		69
52	Unidentified		124
55	Unidentified		3
56	Unidentified		862
66	Unidentified		46

[a] a: Low-resolution GC-MS; b: retention-time comparison using PS-089 column; c: retention-time comparison using OV-240 column; d: CI(CH_4)-MS; e: DMDS derivatization; f: reduction with $LiAlH_4$; g: unsaturated compounds listed in the order in which they are eluted from the OV-240 column, elution order interchangeable on the PS-089 column within each group of homologs; h: not observed with OV-240 column.

chiral constituents of the exocrine secretions of various South African antelope species will be dealt with in a future publication.

It is often difficult to differentiate between saturated and unsaturated long-chain alcohols, formates, and alkenes, especially if they are present in such small quantities that it is difficult to obtain pure spectra from a GC-MS analysis. These spectra often also lack diagnostic ions in the higher mass ranges. Furthermore, the preparative GC isolation of constituents is not feasible if they are present in low concentrations and only a limited quantity of secretion is available. Valuable diagnostic information was, however, obtained by lithium aluminum hydride reduction of the whole extract of the secretion of the male, which left some constituents intact, whereas carbonyl compounds, esters, etc., were reduced to compounds that coeluted with the reduction products of other reducible constituents. Information on the contribution of this experiment to the identification of some of the constituents of the secretion is included in Table 1.

Quantitative extraction of the volatile organic material from the secretion was not possible with small quantities of solvent. The widely varying quantities of secretion collected from individual males furthermore contained considerable proportions of water, as well as heavy material that does not pass through the GC column. It is, therefore, impossible to determine the average quantitative composition of the preorbital secretions of males with even reasonable accuracy. The quantitative results in Table 1 are nevertheless included to give at least an approximate indication of the quantities of most of the compounds present in a relatively large sample of secretion collected from a single male.

The suni is the fifth of several small South African antelope species belonging to the tribe Neotragini of which the chemical composition of the preorbital secretion

TABLE 2. COMPOUND TYPES IDENTIFIED IN PREORBITAL SECRETIONS OF ANTELOPE OF TRIBE NEOTRAGINI

Compound types	Carbon numbers of compounds and other structural information				
	Male and female grysbok, *Raphicerus melanotis*	Male and female steenbok, *R. campestris*	Male oribi, *Ourebia orebi*	Male suni, *Neotragus moschatus*	Male klipspringer, *Oreotragus oreotragus*
n-Alkanes		8–10,12		15,17,19,21,23	
Isoalkanes				15,17,18,19,21–25	
Alkenes				17,19–23 (30)[a]	
Alkan-1-ols	11–15	8–16,20,23–27	9,10		
Alken-1-ols	12–17,19,21,23 (13)[a]	7,10–15 (9)[a]	10,12–14 (4)[a]		
Alkadien-1-ols	13–15,17 (4)[a]	17 (1)[a]	13–16 (4)[a]		
Alkan-1-yl formates	11–25	11–13,20–28	8–16	11–24	
Alken-1-yl formates	13–25 (18)[a]	11–15 (6)[a]	9–15 (8)[a]	13–15,17,19–25 (19)[a]	
Alkadien-1-yl formates	14,15,17,19,21 (5)[a]		12–18 (7)[a]		
Alkan-1-yl acetates		13,15,16	8–17		
Alken-1-yl acetates		13,15,19,21,23,24 (6)[a]	10–17 (10)[a]		
Alkadien-1-yl acetates			13–18 (6)[a]		
Alkanals	12,13	6,7,9	9–13	6–9	
Alkenals	13 (1)[a]		11–14 (4)[a]		
Alkadienals		10 (2)[a]	13–16 (4)[a]		
Cycloalkanones		16–21			

Alkanoic acids	14–18,20	4,8,10,12,14–16,18,20	16–18	5–8,16,18	
Alkenoic acids		12–14,16,18 (5)[a]			
Alkadienoic acids		18 (1)[a]			
Alkan-4-olides (γ-lactones)	15–18,20				
Alkan-5-olides (δ-lactones)	16				
1-Hydroxyalk-2-yl acetates		18,20–22			
2-Hydroxyalk-1-yl acetates	18,20	17,18,20–23			
1-Hydroxyalk-2-yl butanoates		18,20,22			
2-Hydroxyalk-1-yl butanoates		14,16,18,20,22			
Miscellaneous	Heptadec-1-yl methyl sulfide	Isopropyl tetradecanoate	13-Methyl-(*Z*)-8-pentadecen-1-yl formate	Benzoic acid	3-Pentanone
	Methyl nonadec-1-yl sulfide	Isopropyl hexadecanoate		Cholesterol	4-Methyl-2-pentanone
		2-Methylbutanoic acid			
		3-Methylbutanoic acid			
		5-Methyl-3-hexanol			5-Methyl-3-hexanone
		Limonene			4-Methyl-3-hexanone
		Squalene			Ethyl propanoate
		Cholesterol			2-Methylpropyl acetate
		α-Tocopherol			Ethyl-3-methyl butanoate
					2-Methylpropyl propanoate

[a] Total number of double-bond positional and configurational isomers.

has been investigated. Of these antelope, the male klipspringer, *Oreotragus oreotragus*, (Burger et al., 1997) is unique in that the male preorbital secretion contains only eight short-chain, volatile organic compounds: four ketones and four esters. The female preorbital secretion of the klipspringer has so far not been investigated. As mentioned in the introduction, female oribi, *Ourebia orebi* (Mo et al., 1995), do not produce any preorbital secretion, although they possess an external structure that resembles that of the preorbital gland of the male. The preorbital secretions of male and female grysbok, *Raphicerus melanotis*, (Burger et al., 1996) and steenbok, *R. campestris*, (Burger et al., 1999b) have also been reasonably well characterized. Male and female secretions of the two *Raphicerus* species are qualitatively identical. Perhaps the most remarkable feature of members of this tribe is the large number of long-chain saturated and unsaturated formic acid esters present in all secretions investigated so far, with the exception of the klipspringer's preorbital secretion. The results from members of the tribe Neotragini are summarized in Table 2. Due to the large number of double-bond positional and configurational isomers present in some members of this tribe, it is impossible to include all relevant information in Table 2. However, there does not seem to be any indication that the position of double bonds in the unsaturated constituents and their stereochemistry could play a part in the territorial behavior of these animals.

One aspect that merits consideration in future research is the possibility that some constituents of the secretions are not secreted by the preorbital glands but are produced by different strains of the same bacterium or by different bacteria present in the glandular structures of the animals. This is supported by the observation that the secretions are more complex in the members of the tribe that produce secretion only very slowly, whereas the secretion of the male klipspringer, produced at a rate of about 5 mg/min, contains only eight simple compounds. In this case, microbial action probably cannot add to the secretion's complexity before it is deposited as a territorial mark and dries out.

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A NOVEL FUNCTION OF THE TRITERPENE SQUALENE IN A TRITROPHIC SYSTEM

ANNA DUTTON,[1,3] LETIZIA MATTIACCI,[1] RENATO AMADÒ,[2] and SILVIA DORN[1,*]

[1]*Institute of Plant Sciences/Applied Entomology*
Swiss Federal Institute of Technology (ETH)
Clausiusstr. 25/NW, CH-8092 Zürich, Switzerland

[2]*Institute of Food Science*
Swiss Federal Institute of Technology (ETH)
Schmelzbergstr. 9/LFO, CH-8092 Zürich, Switzerland

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Abstract—Changes in apple leaf chemistry after infestation by leafminers and their effect on both host location and host habitat location of the generalist parasitoid *Pholetesor bicolor* were investigated. Chemical analysis of leaf solvent extracts from healthy and leafminer-damaged leaves revealed that herbivory increased the amount of the triterpene squalene ($C_{30}H_{50}$), whereas quantities of all other identified compounds were similar in both plant treatments. To assess the response of parasitoids to host location cues, contact bioassays were conducted with naïve females. Results showed that parasitoids performed a characteristic ovipositional probing more often on the mine-damaged than on the healthy leaf. This behavior was triggered by a hexane extract of the mine-damaged leaf, but not by a healthy leaf extract. A synthetic mixture of the compounds identified in the extract triggered a similar response. A mixture devoid of squalene was not active, whereas squalene alone elicited the probing behavior. To assess the use of the identified compounds in habitat location, Y-tube olfactometer experiments were conducted with naïve and experienced females. Results showed that squalene is not involved in habitat location and has no priming effect on *P. bicolor*. While other triterpenes are known to mediate habitat location of parasitoids, this is the first report in which a plant triterpene is shown to mediate host location of a parasitoid. The biological and ecological functions of squalene on all three trophic levels are discussed.

Key Words—Squalene, semiochemicals, foraging behavior, leafminer parasitoid, apple plant, tritrophic.

*To whom correspondence should be addressed. E-mail: silvia.dorn@ipw.agrl.ethz.ch
[3]Present address: Eidgenössische Forschungsanstalt für Agrarökologie und Landbau, Reckenholzstrasse 191, CH-8046 Zürich, Switzerland.

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INTRODUCTION

Upon insect herbivory, plants generally activate biochemical defenses. These defenses may act directly against herbivores (reviewed by Karban and Baldwin 1997) and/or elicit chemical signals that may attract natural enemies of the herbivore (reviewed by Price 1997). Several plant compounds induced by herbivore feeding have been identified and are most often used by parasitoids during the initial stages of host selection, i.e., for habitat location before landing (Rutledge, 1996). It is relatively unknown how parasitoids use plant chemicals after landing on an infested plant. In fact later stages of host selection, including host location, host acceptance, and oviposition (*sensu* Vinson, 1984) are most often mediated by semiochemicals directly produced by the arthropod host (Vinson, 1991; Vet and Dicke, 1992). There seem to be exceptions where plant derived chemical cues mediate host location of parasitoids. This includes foraging of parasitoids for non-concealed (Horikoshi et al., 1997) as well as concealed hosts (Keller and Horne, 1993; Horikoshi et al., 1997; Mattiacci et al., 1999).

Endophytic hosts such as leafminers impose a particular challenge for natural enemies. Parasitoids searching on a leaf for concealed hosts do not have direct contact with the herbivore. This investigation deals with the chemical signals involved in host location of *Pholetesor bicolor* Ness (Hymenoptera: Braconidae). This endoparasitoid belongs to a large community of natural enemies that parasitize apple leafminers of the genus *Phyllonorycter* (Lepidoptera: Gracillariidae). In Hungarian orchards, *P. bicolor* is considered a primary parasitoid of apple leafminers (Balázs et al., 1996). Because *P. bicolor* is well synchronized with its host (Balázs, 1992), it can be considered a potential biological control agent of *Phyllonorycter* species that cause economic damage in several major apple growing regions of the world (Dorn et al., 1999).

In a recent study on host location of *P. bicolor*, we obtained results indicating an innate response of parasitoids to the herbivore-damaged leaf, but not to the larva or host-derived material (Dutton et al., 2000). The response could be elicited by exposing females to a synthetic mixture of *n*-alkanes and squalene, which were identified from the herbivore-damaged mine area. *n*-Alkanes are important chemicals in both habitat and host location for various parasitoid species (Rutledge, 1996). However, in this system it remains unclear whether these compounds are produced by the plant after leafminer herbivory, which specific compound(s) are used by the parasitoid for host location, and whether they are used by the parasitoid as chemical cues for habitat location.

Habitat location of *P. bicolor* involves olfactory cues emitted by infested leaves (Dorn et al., 1999). Given that this parasitoid has no direct contact with the host upon landing on a leaf, it is possible that similar compounds mediate both habitat and host location. Furthermore, as a generalist parasitoid, *P. bicolor* may benefit from a previous contact exposure to the leaf enclosing its endophytic host

for the subsequent location of leafminers in the same habitat. Indeed, experience is known to increase parasitoid responses towards certain stimuli (i.e., priming) or even generate new ones (i.e., associative learning). For example, previous contact exposure to innately recognized semiochemicals enhances the searching ability of parasitoids used for biological control in greenhouses and the field (Hare et al., 1997; Grasswitz, 1998). *P. bicolor* prefers volatiles emitted by infested over noninfested plants only after prior exposure to plants damaged by leafminers (Lengwiler et al., 1994; Dorn et al., 1999). Presently, the effect of exposure to innately recognized contact cues on the subsequent habitat location of this parasitoid is unknown.

This study was designed to: (1) investigate the chemical response of apple leaves to leafminer herbivory, (2) identify the substance(s) mediating host location of *P. bicolor,* (3) assess the role of apple leaf chemicals for both host and habitat location, and (4) determine the impact of experience on contact cues for the subsequent parasitoid habitat location.

METHODS AND MATERIALS

Plant and Insect Rearing

Two-month-old apple seedlings (*Malus sylvestris* cv. Golden Delicious, Rosaceae) were used for rearing of insects and for preparation of leaf chemical extracts. Plants were grown in climatic chambers at 25°C daylight and 17°C night, 70% relative humidity and 16 L : 8 D photoperiod.

Insects were collected in apple orchards in South Tyrol (northern Italy). Parasitoids were reared in individual cages on four to five apple seedlings heavily infested with sap-feeder leafminers. At pupation, leaves were removed from plants and introduced in a separate cage in which the parasitoids were allowed to emerge. We used three to six-day-old honey-fed female parasitoids for all bioassays. Host location bioassays were conducted with mated naïve females, whereas habitat location experiments were conducted with females that were mated and then given an experience (see details below).

Plant Response to Herbivory

Leaf extracts were obtained by soaking overnight 45-50 apple leaves with 100 mines (2 mines per leaf) in 200 ml hexane. These herbivore-infested apple leaves are referred to as mine-damaged leaves. The hexane extract was filtered through 10 g silica gel (Supelco) that had been previously conditioned with 50 ml hexane, and concentrated first with a rotary evaporator (32 ± 3°C) and then under a stream of nitrogen (purity 99.96%) to 10 μl. The same extraction procedure was applied to healthy leaves that were of similar age and had the same leaf area surface as those with mines. Leaf area was measured with an area meter instrument (Licor 300A).

Ten separate sample extracts of each treatment (mine-damaged and healthy) were analyzed with an HP 5890 Series II Plus gas chromatograph, equipped with a flame ionization detector (300°C) and a nonpolar HP-1 (Crosslink Methyl Silicone Gum) capillary column (30 m × 0.25 mm × 0.25-μm film thickness). The oven temperature program was held at 100°C for 2 min, then increased to 300°C at a rate of 6°C/min, and held at 300°C for 15 min. The split/splitless injector (250°C) was operated in splitless mode throughout the analyses. The carrier gas was helium (purity 99.96%) at a pressure of 108 kPa (constant pressure). Samples of 1 μl were injected. To evaluate quantitative differences among treatments for the individual compounds identified, peak areas were analyzed using a *t* test with the appropriate Bartlett's test for homogeneity and Kolmogorov-Smirnov test for normality (Sokal and Rohlf, 1981). To quantify the compounds found in mine-damaged leaves and healthy leaves, three additional extracts of each treatment were analyzed with an internal standard (100 ng octylbenzene/μl). The quantities of each compound were calculated based on GC peak areas relative to the internal standard. A response factor for each identified compound in relation to the internal standard was calculated. Identification of compounds by coinjection with authentic standards was performed with gas chromatography–mass spectrometry (GC-MS). Analyses were done using a HP 6890 Series GC connected to an HP 5973 electron-impact (EI) MS. The chromatographic conditions were kept as described above. The MS temperature parameters were: 230°C at the source, 150°C at the quadruple, and 280° at the interface. The masses were swept at 23–600 mass units at a rate of 2.6 scans/sec.

Parasitoid Response to Contact Chemical Cues for Host Location

All behavioral contact assays were performed in a single choice Petri dish (10 cm diam.) arena. Parasitoids were placed individually in glass vials (2 cm diam. × 4 cm high) and the open end of the vial was positioned over the plant cue sources or over an extract treated filter-paper (1.3 cm diam.). Females that were observed performing the characteristic thrusting of the ovipositor, from here on referred to as probing, on the filter-paper for more that 5 sec were considered to respond positively to the treatment. Observations were conducted under laboratory conditions at 23 ± 2°C, 50 ± 10% relative humidity and 1000 ± 500 lux. Each treatment was bioassayed simultaneously with a control, and for statistical analysis a χ^2 test was performed between treatments and controls.

Experiment 1. Response to Healthy and Mine-Damaged Leaf Material. In order to elucidate whether the source of active compounds eliciting ovipositional probing of *P. bicolor* is specific to herbivore damage, female parasitoids were offered the following cue sources: (1) a 1.2-cm^2 piece of healthy lower-leaf epidermis (healthy), (2) a 1.2-cm^2 piece of lower-leaf epidermis damaged by one larva (mine-damaged), from which the larva and its by-products were

carefully removed, or (3) an empty Petri dish (control). The leaf area offered corresponded to the size of a fully developed mine. This reference area was used throughout the work for quantifying the compounds extracted from leaves and for calculating the concentrations of the synthetic mixture used in subsequent experiments.

The behavioral parameters recorded were the time spent walking, standing, and probing on the cue source offered. The parameters were recorded over a 20-min period with The Observer computer software (Noldus, 1991). Each behavioral parameter was analyzed by Kruskal-Wallis ANOVA followed by pairwise multiple comparisons between treatments using the Student-Newman-Keuls method (Sokal and Rohlf, 1981).

Experiment 2. Response to Healthy and Mine-Damaged Leaf Extracts. In order to examine whether host location is elicited by plant chemical cues, solvent extracts from both healthy and mine-damaged leaves were tested. Leaf epidermis from each plant treatment was carefully removed with tweezers and soaked separately in hexane (purity 99.7%) at 4°C for 24hr. The extract concentration applied to the filter-paper was made so that 10 μl were equivalent to 1.2 cm^2 mine-damaged or healthy leaf. Hexane alone was applied to the filter paper as a control.

Experiment 3. Response to a Mixture of n-Alkanes and/or Squalene. A mixture of the synthetic *n*-alkanes *n*-C_{27}, *n*-C_{29}, *n*-C_{30}, *n*-C_{31}, *n*-C_{32}, *n*-C_{33}, and squalene ($C_{30}H_{50}$) (Fluka Chemie AG, purity $\geq$ 99.5%) at quantities equivalent to 1.2 cm^2 mine-damaged leaf (see Table 1) was tested against a control (hexane). A dose–response test was carried out with the same reference mixture using a dilution factor of 10. The mixture of *n*-alkanes without squalene was tested, and subsequently a dose–response test of squalene was performed.

Parasitoid Response to Host Location Cues for Habitat Location

Two Y-tube olfactometer bioassays were conducted to examine: (1) whether the identified chemical cues identified in mine-damaged leaves are used by *P. bicolor* as olfactory stimuli for habitat location, and (2) whether a previous contact exposure to innately recognize plant-derived cues enhance habitat location of *P. bicolor* through priming (Hare et al., 1997).

The olfactometer consisted of a glass Y-shaped tube. Odor sources were placed in glass chambers (27 cm long $\times$ 2.5 cm diam.) which directly fitted to each of the two Y-tube arms. Humidified air (300 ml/min) entering the odor source chamber was filtered through a charcoal filter. Parasitoids were individually introduced in the central arm of the olfactometer and given a maximum of 10 min to make a choice for one of the two olfactometer arms. A choice was recorded when the female had crossed a line marked 20 cm after the junction of the two arms. Responses of *P. bicolor* in the olfactometer were analyzed with a χ^2 test

with null hypothesis of a 50 : 50 distribution over the two odor sources. Females that did not respond during the 10-min observation were not included in the analysis. To avoid a bias in arm preference, odor sources were reversed after every third tested female. The environmental conditions in the bioassay room were 23 ± 2°C, 50 ± 5% relative humidity and 1200 ± 50 lux provided with artificial light.

Experiment 4. Squalene and n-Alkanes as Olfactory Stimuli. In the Y-tube olfactometer females were given the choice between the synthetic mixture of squalene and *n*-alkanes identified in the mine-damaged leaves and pure solvent as control (blank). The mixture of compounds was tested at a concentration equivalent to 1.2 cm^2 mine-damaged leaf surface and at three higher concentrations (dilution factor of 10). The compounds in hexane were applied to a Teflon septum (1.3 cm diam.) and placed in one arm of the Y-tube. A similar septum with hexane (blank) was placed in the other arm of the Y-tube.

Prior to being tested, wasps were given 25 ± 5 min contact experience on leafminer-infested plants. Preliminary experiments showed that naïve parasitoids are not attracted to mine-damaged plants. Experienced wasps were placed individually in vials for 30 min before they were tested.

Experiment 5. Squalene and n-Alkanes as Priming Stimuli. In the Y-tube olfactometer, females were given the choice between one infested apple leaf containing five mines (mined leaf) or clean air (blank). Wasps were given either no experience (naïve) or a 25 ± 5 min contact experience on one of the following treatments: (1) a leafminer infested plant, (2) the hexane extract of mine-damaged leaf, (3) the synthetic mixture of squalene and *n*-alkanes, or (4) a healthy plant. The contact experience with leafminer infested or healthy plants was provided as in the previous experiment (see above). Experience on the leaf extract or the synthetic mixture was provided by offering the solution in a filter-paper as in the host location bioassays.

RESULTS

Plant Response to Herbivory. Eight major compounds were identified by GC-MS in extracts from apple leaves infested with the leafminer *P. pomonella* and from healthy apple leaves (Table 1). Among them, only squalene ($C_{30}H_{50}$) was found in the mine-damaged leaves in larger quantities than in the healthy leaves (Student's *t* test, t value $= 2.6$; $df = 9$; $P = 0.03$). Only one minor peak was not identified, and all other compounds consisted of long-chain hydrocarbons (*n*-C_{27}, *n*-C_{29}, *n*-C_{30}, *n*-C_{31}, *n*-C_{32}, *n*-C_{33}).

Experiment 1. Response to Healthy and Mine-Damaged Leaf Material. The behavior of *P. bicolor* offered mine-damaged or healthy leaf material is depicted in Figure 1. Wasps spent more time walking, standing, and probing on the mine-damaged leaf ($P < 0.05$, Kruskal-Wallis ANOVA) than on the healthy leaf.

TABLE 1. COMPOUNDS EXTRACTED WITH HEXANE FROM *Phyllonorycter pomonella* HERBIVORE-DAMAGED AND HEALTHY APPLE LEAVES (*Malus sylvestris*)[a]

Compound	Leaf		
	Herbivore-damaged	Healthy	*P*
Heptacosane *n*-C_{27}	10.7 ± 0.8	8.2 ± 0.7	n.s
Unknown	6.3 ± 1.1	4.0 ± 0.9	n.s
Squalene $C_{30}H_{50}$	32.3 ± 13.1	12.2 ± 4.7	<0.05
Nonacosane *n*-C_{29}	91.3 ± 8.9	77.4 ± 11.3	n.s
Triacontane *n*-C_{30}	8.7 ± 1.2	7.3 ± 0.8	n.s
Hentriacontan *n*-C_{31}	251.6 ± 36.3	228.8 ± 18.8	n.s
Dotriacontane *n*-C_{32}	5.9 ± 0.9	5.6 ± 0.6	n.s
Tritriacontane *n*-C_{33}	34.0 ± 5.5	34.2 ± 1.7	n.s
Total	440.8 ± 67.6	377.8 ± 39.5	n.s

[a] [Average amount (ng $\pm$ SE) extracted from 1.2 cm^2 leaf surface (*t* test between treatments, $N = 10$)].

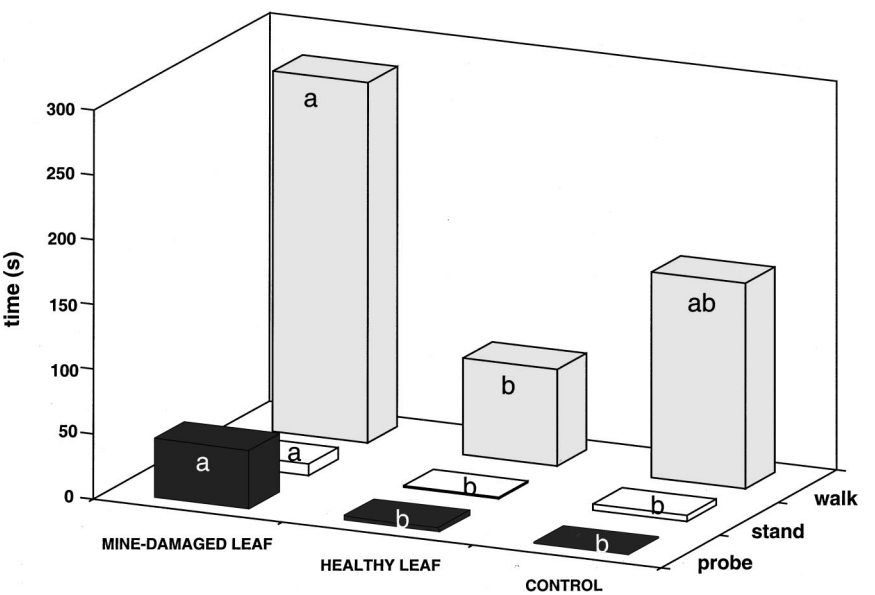


FIG. 1. Walking, standing, and probing activity of the parasitoid *P. bicolor* on mine-damaged and healthy apple leaf epidermis. Statistical differences in time duration spent for each activity on the different treatments are shown with different letters (Kruskal-Wallis ANOVA, $P < 0.05$).

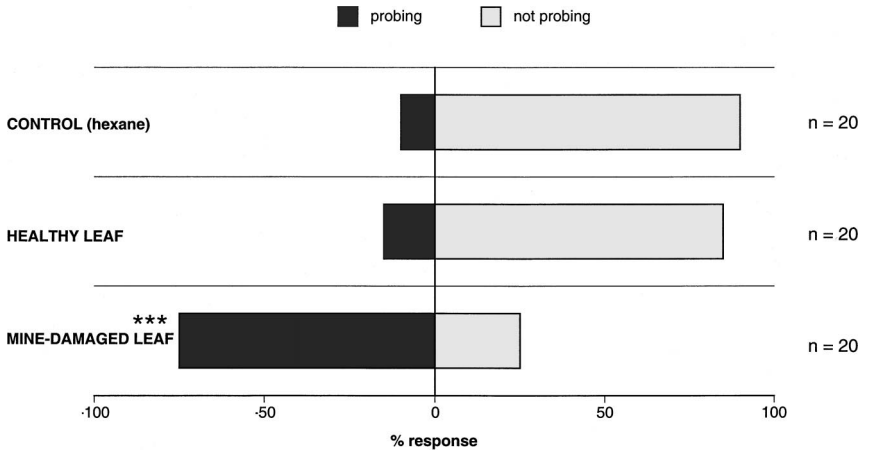


FIG. 2. Ovipositional probing response of *P. bicolor* females to hexane extracts of mine-damaged and healthy leaf epidermis. Mined-damaged vs. control: $^{***}\chi^2 = 14.7$, $df = 1$, $P < 0.001$.

In addition, no significant differences for probing and standing were observed between the healthy leaf and an empty arena (control). Only walking was similar on the mine-damaged leaf and on the control.

Experiment 2. Response to Healthy and Mine-Damaged Leaf Extracts. The ovipositional probing behavior of *P. bicolor* was triggered only by the hexane extract from mine-damaged leaves ($\chi^2 = 14.7$; $df = 1$; $P < 0.001$) (Figure 2). In contrast, the response of females to the extract from healthy leaves was similar to that of the control solvent, and substantially lower than the response to the mine-damaged leaves ($\chi^2 = 12.2$; $df = 1$; $P < 0.001$).

Experiment 3. Response to a Mixture of n-Alkanes and/or Squalene. The percent of females responding in the contact bioassay to the mixtures of synthetic compounds identified from the mine-damaged leaves is shown in Table 2. The mixture of *n*-alkanes and squalene elicited ovipositional probing behavior of *P. bicolor* at concentrations corresponding to quantities found in 1.2 cm^2 = 1 × ($\chi^2 = 20.1$, $P < 0.001$), 12 cm^2 = 10 × ($\chi^2 = 11.8$, $P < 0.001$), and 120 cm^2 = 100 × ($\chi^2 = 15.1$, $P < 0.001$) of mine-damaged leaf. The mixture of *n*-alkanes (1×) devoid of squalene did not trigger a response ($\chi^2 = 2.5$, $P > 0.05$). In contrast, squalene alone triggered a response over a range of concentrations from 30 ng to 3.1 μg. These quantities corresponded to those found in 1.2, 12, and 120 cm^2 surface area of mine-damaged leaves. The strongest response was obtained when females were offered 30 ng of squalene. This amount of squalene elicited a response that was significantly higher than to the solvent control, within the first 20 min of the bioassay.

TABLE 2. OVIPOSITIONAL PROBING RESPONSE OF FEMALE *Pholetesor bicolor* TO SYNTHETIC COMPOUNDS IDENTIFIED FROM *Phyllonorycter pomonella* HERBIVORE DAMAGED APPLE (*Malus sylvestris*) LEAVES[a]

Treatment	*n*	% females probing					
		20 min	χ^2	*P*	90 min	χ^2	*P*
Synthetic mixture (all compounds)							
0.1×	30	10	0.8	n.s.	23	2.9	n.s.
1×	30	30	5.1	≤0.05	70	20.1	≤0.001
10×	30	20	2.6	n.s.	50	11.8	≤0.001
100×	30	13	0.2	n.s.	57	15.1	≤0.001
1000×	30	7	0	n.s.	27	2.9	n.s.
synthetic mixture (1×) (excluding squalene)	30	3	0	n.s.	30	2.5	n.s.
Squalene (ng)							
3	30	7	0	n.s.	27	2.9	n.s.
15	30	7	0	n.s.	33	3.5	n.s.
30	30	30	4.0	≤0.05	63	16.1	≤0.001
310	30	13	0.2	n.s.	57	15.1	≤0.001
3100	30	20	2.3	n.s.	57	12.7	≤0.001
31000	30	7	0	n.s.	27	2.9	n.s.

[a][Concentration 1× corresponds to that given in Table 1 offered on a 1.3-cm^2 filter paper. The total observation time for each female was 90 min. χ^2 performed between treatment and control (hexane).]

Experiment 4. Squalene and n-Alkanes as Olfactory Stimuli. In the Y-tube olfactometer bioassay, no response of females towards the mixture of squalene and *n*-alkanes was observed (Figure 3), regardless of the concentration offered. This indicates that squalene is not used by *P. bicolor* as a chemical cue in habitat location.

Experiment 5. Squalene and n-Alkanes as Priming Stimuli. In the Y-tube, only females that had been given a previous contact experience on leafminer damaged plants preferred the volatiles emitted by the mine-damaged leaf versus the control ($\chi^2 = 15$; $P < 0.001$) (Figure 4). Neither a previous contact experience with the mine-damaged leaf extract nor a contact with the synthetic mixture, induced a preference towards the odor of the mined leaf.

DISCUSSION

Apple leaves infested by leafminers produce the triterpene squalene ($C_{30}H_{50}$) in quantities higher than noninfested leaves. Squalene elicits a characteristic ovipositional probing behavior of naïve *P. bicolor* which demonstrates that this chemical

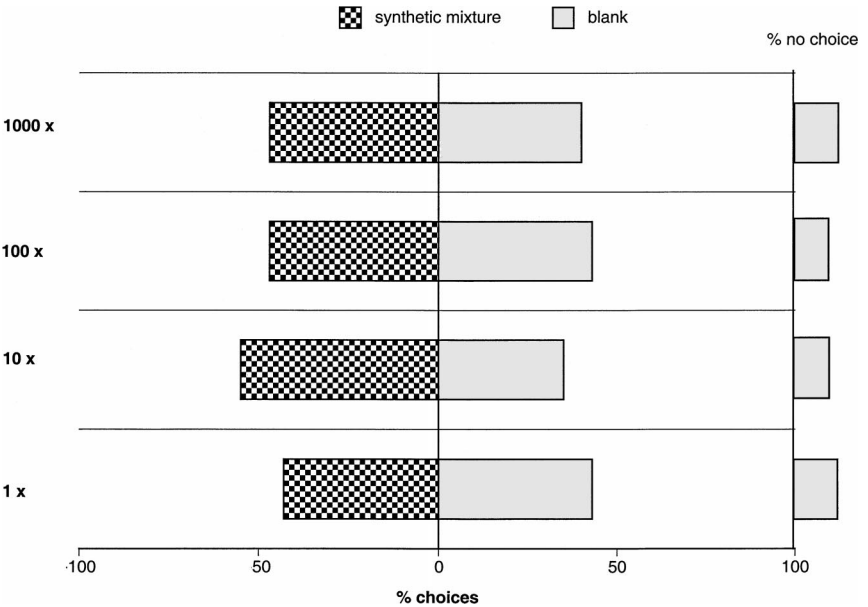


FIG. 3. Response of *P. bicolor* to the synthetic mixture of squalene and *n*-alkanes in the Y-tube olfactometer. Concentrations of synthetic mixture are indicated on the left side (dilution factor of 10). 1× corresponds to 1.2 cm^2 leaf area surface of mine-damaged leaf (see Table 1 for quantities). Percent females making no choice is indicated on the right side ($N = 30$ for each test).

is innately recognized and used by the parasitoid for host location after landing on a host-infested patch. Given that squalene cannot be synthesized by arthropods (Downer, 1985), we conclude that this compound is of plant origin. Its increased production seems to be induced by *Phyllonorycter pomonella's* herbivory. Previous reports have documented herbivore induction of terpenes in plants, and their use by parasitoids and predators for habitat location (for reviews see Rutledge, 1996 and Dicke, 1994). However, this specific triterpene has not been reported as a plant-derived semiochemical for parasitoids. Squalene in this system can be defined as a contact plant synomone for the parasitoid (sensu Dicke and Sabelis, 1988).

To our knowledge, this is the first record of squalene being extracted from apple leaves. The most closely related plant species from which squalene has been extracted is *Prunus persica* (L.) Batsch (Lin and Lin, 1992). Squalene is widely found in both plant and animal tissue, being a basic intermediate metabolite for the biosynthesis of sterols and triterpenes (Bonner, 1965). From the genesis of squalene through the mevalonate → geranyl pyrophosphate → farnesyl pyrophosphate

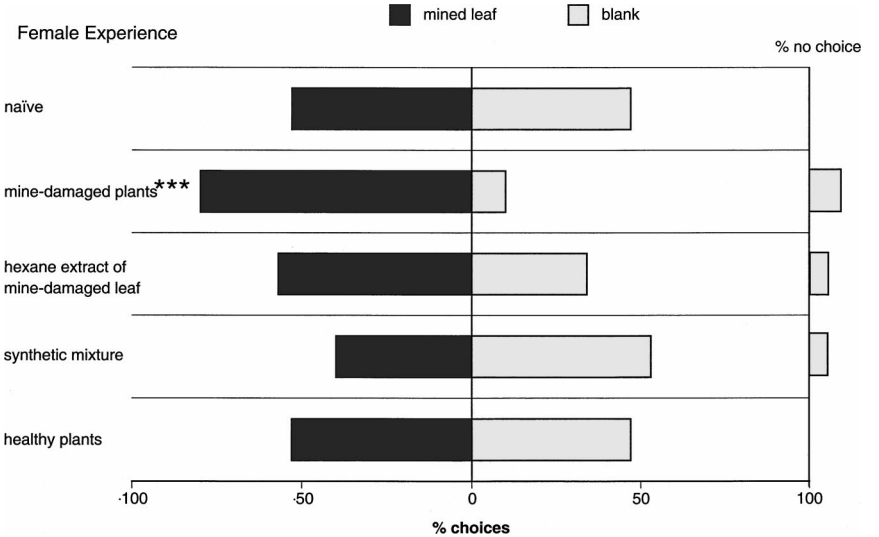


FIG. 4. Response of *P. bicolor* females to mine-damaged leaf in Y-tube olfactometer. Type of experience is indicated on left side. Percent females making no choice is indicated on the right side. Asterisks indicate a significant difference within a choice test: χ^2, $^{***}P < 0.001$ ($N = 30$ for each test).

pathway, and the subsequent steps leading to sterol production (squalene epoxidase → squalene 2,3-oxide), it is evident that squalene is an essential compound regulating plant growth (Yates et al., 1991; Simmen and Gisi, 1995). The increase of squalene in organisms such as plants, fungi, and mammals has been correlated to the exogenous inhibition of squalene epoxidase (Petranyi et al., 1984; Ryder and Dupont, 1985; Yates et al., 1991; Simmen and Gisi, 1995). The inhibition of squalene epoxidase in these organisms is shown to constrain the biosynthesis of sterols and consequently lead to growth inhibition. In our apple plant system, we observed both an increase of squalene (Table 1), and a decrease of plant growth in mine-damaged seedlings as compared to healthy seedlings (A. Dutton, personal observation). We can, therefore, hypothesize a possible direct effect of squalene on the first trophic level.

Given that squalene is an intermediate in the biosynthesis of other triterpenoids and sesquiterpenoids, it might indirectly affect the second trophic level. In fact, different plant species produce sesquiterpenoids and triterpenoids as defense compounds against pathogens (phytoalexins) and/or herbivores (Threlfall and Whitehead, 1988; Gershenzon and Croteau, 1991; Zook and Kuc, 1991). Although squalene itself has not been reported as a phytoalexin or as a toxin to herbivores, its involvement in phytoalexin biosynthesis in plant cell cultures after

fungal infection is known (Van Der Heijden et al., 1989). It would be of interest to determine if squalene has a behavioral effect on the leafminer or on other herbivore species.

Our results indicate a behavioral effect of squalene on the third trophic level. The almost threefold increase of squalene in mine-damaged leaves, with a mean value of 32 ng/1.2 cm^2 (Table 1) triggered the observed ovipositional probing behavior of the parasitic wasp *P. bicolor* (Table 2). A slightly higher amount of squalene was extracted from the mined leaf area (mean = 37 ng/1.2cm^2) (Dutton et al., 2000) as that extracted from the whole leaf (mean = 32 ng/1.2cm^2, Table 1). This indicates a possible localized increase in concentration of this substance at the site of herbivory. This finding coincides with our behavioral observation that parasitoids probe on the mined leaf area and not on healthy tissue. A similar localized response of plant compounds upon herbivory and a related parasitoid response were recently shown in the crucifer-*Pieris*-*Cotesia* tritrophic system (Horikoshi et al., 1997).

Several terpenes are known to be involved in habitat and host location of parasitoids (Rutledge, 1996). To our knowledge, this is the first report of squalene being involved in host location of a parasitoid. Behavioral effects of squalene in interactions among other organisms are known. For example, ticks of the genus *Dermacentor* ingest squalene from mammal blood and use the substance as an allomone to protect themselves against ant attack (Yoder et al., 1993). Furthermore, pollinating bats use squalene as a flower attractant (Ecroyd et al., 1995).

Enhanced responsiveness of parasitoids after a brief exposure to innately recognized cues has been demonstrated previously (Turlings et al., 1989; Lewis and Martin, 1990; Hare et al., 1997). In our system, exposing *P. bicolor* females to innately recognized cues such as squalene did not result in a subsequent attraction to leafminer infested leaves (Figure 5). However, we cannot rule out the possibility that this substance is used in associative learning (Vet et al., 1995). If such were the case, the parasitoid would need a previous exposure to the contact cue in association with plant volatiles. This can be supported by the fact that parasitoids exposed to contact and volatile cues (i.e., females given a contact experience on leafminer infested plants) subsequently were attracted to herbivore–plant volatiles.

In the Y-tube olfactometer experiments, we could not demonstrate a habitat location response to the mixture of squalene and *n*-alkanes. However, in a previous study (Lengwiler et al., 1994) and in the present experiments (Figure 4), we observed a significant attraction of parasitoids to herbivore induced leaf volatiles. It seems evident, therefore, that the volatile and contact cues used by *P. bicolor* for habitat and host location are different. In both cases the cues are of plant origin.

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SILPHINENE SESQUITERPENES AS MODEL INSECT ANTIFEEDANTS

AZUCENA GONZÁLEZ-COLOMA,[1,*] FERNANDO VALENCIA,[1] NURIA MARTÍN,[1] JOSEPH J. HOFFMANN,[2] LOUIS HUTTER,[2] J. ALBERTO MARCO,[3] and MATÍAS REINA[4]

[1]*Centro de Ciencias Medioambientales, CSIC*
Serrano 115-dpdo., 28006 Madrid, Spain

[2]*Southwest Center for Natural Products Research and Commercialization*
University of Arizona
250 E. Valencia Rd., Tucson, Arizona 85706

[3]*Universidad de Valencia*
Departamento de Química Orgánica
Dr. Moliner 50, 46100 Burjassot, Valencia, Spain

[4]*Instituto de Productos Naturales y Agrobiología, CSIC*
Avda. Astrofísico F. Sánchez, 3, 38206 La Laguna, Tenerife, Spain

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Abstract—Silphinene sesquiterpenes are established chrysomelid antifeedants. In this work, nine silphinene analogs, 11β-acetoxy-5α-angeloyloxysilphinen-3-one (**1**), 11β-acetoxy-5α-tigloyloxysilphinen-3-one (**2**), 11β-acetoxy-5α-isobutyryloxysilphinen-3-one (**3**), 11β-hydroxy-5α-angeloyloxysilphinen-3-one (**4**), 11β,5α-dihydroxysilphinen-3-one (**5**), 11β,5α-diacetoxysilphinen-3-one (**6**), 5α,11β-diisobutyryloxysilphinen-3-one (**7**), silphinen-3,5,11-trione (**8**), and *O*-methyl-5-epicantabrenolic acid methyl ester (**10**), and a presilphiperfolane sesquiterpene (**9**) were tested against several divergent insect species, including the lepidopteran *Spodoptera littoralis*, the chrysomelid *Leptinotarsa decemlineata*, and five aphid species, and their antifeedant effects were compared with those of picrotoxinin, a GABA-antagonist, and thymol, an allosteric modulator for insect GABA receptors. All insects tested responded to at least one silphinene analog and/or GABA antagonist. Compound **3** and thymol were effective antifeedants against all species tested except *S. littoralis*, with varying potencies according to their feeding ecologies. The toxicity of these compounds was species-dependent and did not correlate with their antifeedant effect.

Key Words—Silphinene sesquiterpenes, picrotoxinin, thymol, GABA inhibitor, antifeedants, Colorado potato beetle, aphids.

*To whom correspondence should be addressed. E-mail: azu@ccma.csic.es

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INTRODUCTION

The role of insect taste on host-plant acceptance is basic to the understanding of insect–plant interactions (Bernays and Chapman, 1994). In most cases, antifeedant compounds play an important role in host-plant selection by phytophagous insects (Bernays and Chapman, 1977, 1994, 2000; Mitchell, 1994). The molecular basis of insect taste, however, remains unknown.

Gustatory studies carried out on western corn rootworm (*Diabrotica virgifera virgifera* LeConte, WCR) have shown a direct correlation between antifeedant potency and known antagonistic action at GABA (γ-aminobutyric acid)/glycine amino-acid neuroreceptors (Mullin et al., 1992, 1994; Chyb et al., 1995; Eichenseer and Mullin, 1997). These antagonists include isoquinoline and indole alkaloids and sesquiterpenes (picrotoxinin).

Tricyclic silphinene sesquiterpenes such as compounds **1**, **4**, and **5** (Figure 1) are strong antifeedants to the Colorado potato beetle CPB; (*Leptinotarsa decemlineata* Say) (González-Coloma et al., 1995, 1997). A comparative study between the western corn rootworm and CPB included these silphinenes among 15 compounds, such as alkaloids, terpenoids, and phenolics. The silphinene analogs and all the established GABA- and glycinergic compounds tested acted as antifeedants to both insect species, suggesting a shared molecular mechanism for antifeedant taste chemoreception in divergent Chrysomelidae species (Mullin et al., 1997).

Additionally, sesquiterpenes with strong antifeedant effects on *L. decemlineata*, such as drimanes and bisabolanes, are also active feeding inhibitors to the aphid *Myzus persicae* Sulz (Griffith and Pickett, 1980; Caprioli et al., 1987; Gutiérrez et al., 1997), indicating that CPB sesquiterpene antifeedants are good aphid-antifeedant candidates.

Here we studied the antifeedant effects of 10 silphinene sesquiterpenes against several divergent insect species, including the lepidopteran *Spodoptera littoralis* Bois, the chrysomelid *L. decemlineata*, and five aphid species with diverse host adaptations. The molecules tested are shown in Figure 1 and included 11β-acetoxy-5a-angeloyloxysilphinen-3-one (**1**),11β-acetoxy-5α-tigloyloxysilphinen-3-one (**2**),11β-acetoxy-5α-isobutyryloxysilphinen-3-one (**3**),11β-hydroxy-5α-angeloyloxysilphinen-3-one (**4**), 11β,5α-dihydroxysilphinen-3-one (**5**), 11β, 5α-diacetoxysilphinen-3-one (**6**), 5α,11β-diisobutyryloxysilphinen-3-one (**7**), silphinen-3,5,11-trione (**8**), the presilphiperfolane **9**, and silphinene *O*-methyl-5-epicantabrenolic acid methyl ester (**10**). Compounds **1–4**, **9**, and **10** are natural products, and their biological activities have been compared with those of the semisynthetic ones (**5–8**) to establish their structure–activity relationships. Their antifeedant effects have been compared with those of picrotoxinin, an established GABA-gated chloride channel antagonist (Klunk et al., 1983; Ozoe and Matsumura, 1986) and thymol, an allosteric modulator for insect GABA receptors

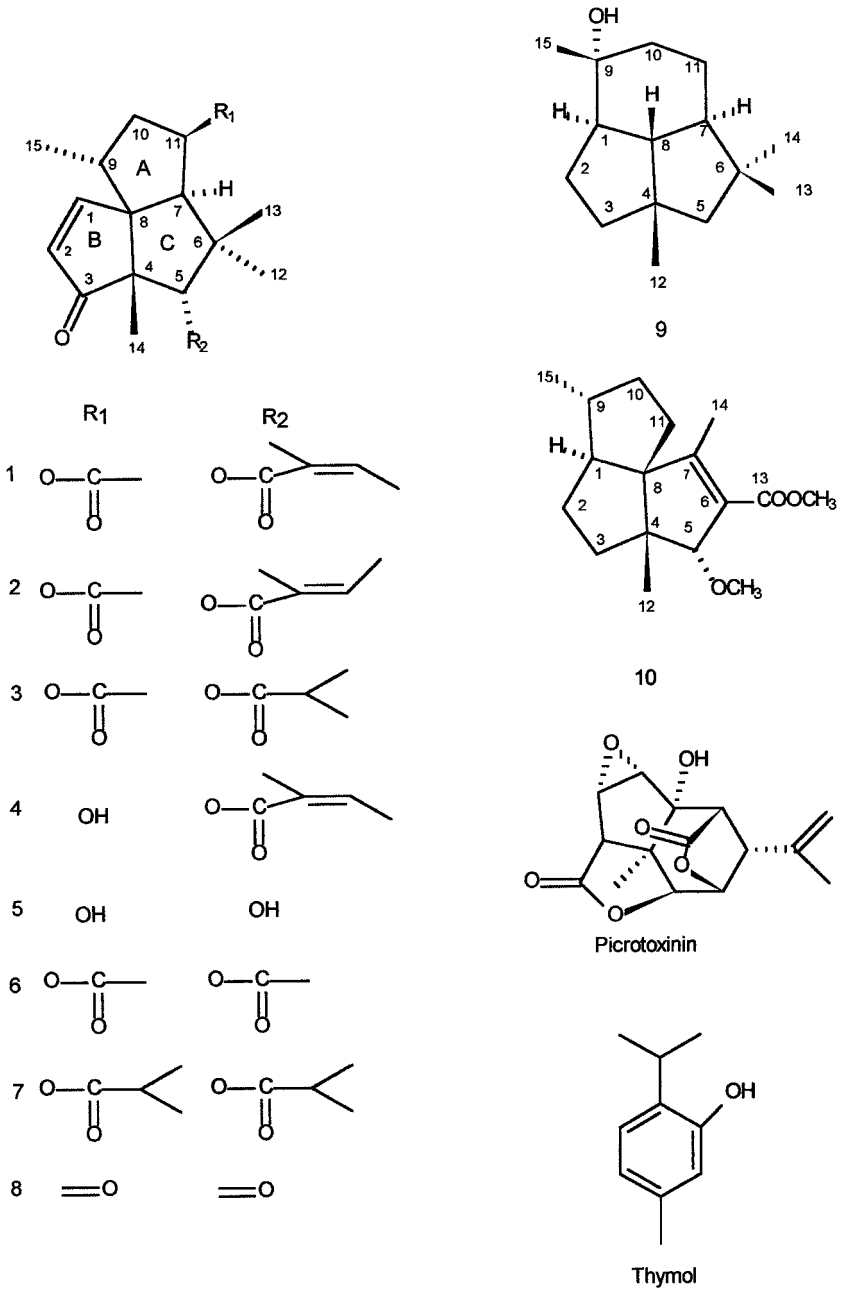


FIG. 1. Molecular structures of compounds **1–10**, picrotoxinin, and thymol.

(Priestley et al., 1999). The sesquiterpene farnesol was included as a positive control for aphid antifeedants (Gutiérrez et al., 1997).

METHODS AND MATERIALS

General Experimental Procedures

NMR spectra were measured on a Bruker AMX2 500-MHz spectrometer with pulsed-field gradient, using the solvent as internal standard ($CDCl_3$, at δ_H 7.26 and δ_C 77.0). Exact mass measurements and electronic impact–mass spectroscopy (EI-MS) results were recorded on an Autospect instrument at 70 eV. HPLCs were carried out on a Gilson-302, equipped with a 115 model UV detector and a Waters Prep-LC 4000 equipped with a diode-array detector. Silica gel from Merck (15111, 7741, 5550) and Aldrich (9,944-3) were used for column chromatography and TLC. Sesquiterpenes were visualized on TLC with a 25% H_2SO_4 solution.

Insects

S. littoralis, *L. decemlineata*, and the aphid colonies (*M. persicae*, *Diuraphis noxia*, *Rhopalosiphum padi*, *Metopolophium dirhodum*, and *Sitobion avenae*) were reared on artificial diet (Poitout and Bues, 1974) and their respective host plants (*Solanum tuberosum*, *Capsicum annuum*, and *Hordeum vulgare*) and maintained at $22 \pm 1^{\circ}$C, >70% relative humidity, and photoperiod of 16L:8D in a growth chamber.

Choice Feeding Assays

These experiments were conducted with *S. littoralis* L6 larvae, adult *L. decemlineata*, and apterous aphid adults. For *S. littoralis* and *L. decemlineata*, each treatment consisted of 5–10 Petri dishes with 2 or 4 insects each as described in González-Coloma et al. (1997). The uneaten leaf disk surfaces were measured according to Escoubas et al. (1993) with a computer interfaced scanner. Percent feeding reduction (%FR) was determined for each Petri dish by the equation $\%FR = [1 - (\text{treatment consumption/control consumption})] \times 100$ (Bentley et al., 1984). For the aphids, each treatment consisted of 20 boxes with 10 insects each as described in Gutiérrez et al. (1997). A settling inhibition index (%SI) was calculated for each compound [$\%SI = 1 - (\%T/\%C) \times 100$, where $\%T$ is percent aphids on treated surface, $\%C$ is percent aphids on control surface] (Gutiérrez et al., 1997). Compounds with FR or SI > 60% at an initial dose of 50 μg/cm^2 were tested in a dose–response experiment to calculate their relative potencies (EC_{50} values, the effective dose for 50% feeding/settling reduction), which was determined from linear regression analysis (%FR or %SI on log dose).

Oral Cannulation

This experiment was performed with preweighed newly emerged *S. littoralis* L6 larvae under the same environmental conditions as above. Each experiment consisted of 20 larvae orally dosed with 20 μg of the test compound in 2 μl DMSO (treatment) or solvent alone (control) as described in González-Coloma et al. (1998). At the end of the experiments (72 hr), larval consumption and growth were calculated on a dry weight basis (see González-Coloma et al., 1998 for details). An analysis of covariance (ANCOVA) was performed on biomass gains and food consumption with initial biomass as covariate (Raubenheimer and Simpson, 1992; Horton and Redak, 1993).

Hemolymph Injection

DMSO solutions of the test compounds (10 μg each per insect) were injected through the metepimeron suture of the thorax of 20 adult *L. decemlineata* beetles as described in González-Coloma et al. (1998). Toxicity symptoms and mortality were recorded up to three days after injection by maintenance of beetles on their respective potato leaf foods. Percent mortality was analyzed with contingency tables and corrected according to Abbott (1925).

Isolation of Compounds

Compounds **1–4** were isolated from *Senecio palmensis* Chr. Sm. (Asteraceae) (collected in Boca Tauce, Tenerife, July 1996, voucher number ORT 36393, Jardín Botánico de La Orotava, Tenerife). Dried aerial parts of *S. palmensis* (2.8 kg) were exhaustively extracted with EtOH. The ethanolic extract (1000 g, 35.7% yield of dry plant weight) was chromatographed on a silica gel (Si gel) vacuum liquid chromatography (VLC) column using an *n*-hexane–EtOAc–MeOH gradient (*n*-hexane–EtOAc, 100:0–50:50) to give fractions A–G.

Fraction D, 14.5 g (1.45% yield, *n*-hexane–EtOAc, 95:5) was subsequently purified by Si gel-VLC, Sephadex LH-20, Si gel-column chromatography (CC), and semiprep normal-phase HPLC chromatography using a 250- $\times$ 20-mm silica column (Inertsil Prep-Sil, 10 μm particle size) at a flow rate of 10 ml/min and an isocratic mixture of Cl_2CH_2–$CNCH_3$ (92:8) to obtain 2.0 g of a mixture of **1** and a minor compound (0.21% yield). Peaks were detected at 254 nm. One hundred milligrams of this mixture were further purified by semiprep reversed-phase HPLC chromatography using a 250- $\times$ 20-mm silica column (Kromasil C18, 5-μm particle size) at a flow rate of 20 ml/min and a $CNCH_3$–H_2O gradient (65:35–100:0) to obtain 56 mg of pure **1** (0.11% yield) and 4 mg of pure **2** (5.3×10^{-3}% yield). Peaks were detected at 235 nm.

Fraction E, 70 mg (1.8 g, 0.06% yield, *n*-hexane–EtOAc, 90:10) was purified by semiprep normal-phase HPLC chromatography using a 250- $\times$ 8-mm silica

column (Nucleosil, 10-μm particle size) at a flow rate of 1.5 ml/min and a *n*-hexane–EtOAc gradient (92:8–90:10) to obtain 10.9 mg of a mixture (0.01% yield). This mixture was further purified by a gradient (Cl_2CH_2–$CNCH_3$, 100:0–96:4) and a subsequent isocratic (Cl_2CH_2–$CNCH_3$, 97:3) HPLC elution to yield compound **3** (6.5 mg, 5.9×10^{-3}% yield). Peaks were detected at 254 nm.

Fraction F, 1.8 g (6.3×10^{-2}% yield, *n*-hexane–EtOAc, 90:10) were subsequently purified by VLC and Si gel CC and eluted with *n*-hexane–EtOAc mixtures. The eluate obtained with *n*-hexane–EtOAc 85:15 afforded 60 mg of **4** (2.1×10^{-3}% yield).

Compound **5** was generated from **1** by chemical hydrolysis (González-Coloma et al., 1997). Compound **5** (100 mg) was acetylated with Ac_2O-pyridine at room temperature for 24 hr to give a residue of 142 mg. This residue was chromatographed over silica-gel to yield compound **6** (128.2 mg, 95.95%). A mixture of **5** (10 mg), pyridine (0.1 ml), and isobutyric anhydride (0.4 ml) was kept at room temperature for 72 hr to afford the reaction product, compound **7** (11.3 mg, 72.1%), after purification over a silica-gel column. A mixture of **5** (10.6 mg), pyridine (0.2 ml), and Cornforth's reagent (0.5 ml) was kept at room temperature for five days. The excess of reagent was decomposed with EtOH, the solvent removed, and the residue chromatographed over silica-gel column eluting with hexane–EtOAc (60:40), to give compound **8** (6.0 mg, 58%).

Compounds **9** and **10** were previously isolated from *Artemisia chamaemelifolia* Vill. (Asteraceae) (Marco et al., 1996) and provided by Prof. J. A. Marco (U. de Valencia, Spain).

Identification of Compounds

Compounds **1**, **4**, and **5** were identified as *11β-acetoxy-5α-angeloyloxysilphinen-3-one, 11β-hydroxy-5α-angeloyloxysilphinen-3-one, and 11β,5α-dihydroxysilphinen-3-one* by comparing their spectra with previously published data (Jakupovic and Abraham, 1985; González-Coloma et al., 1995, 1997).

*11β-Acetoxy-5α-tigloyloxysilphinen-3-one (**2**).* 1.8 mg. oil; EI-MS (70 eV, *m/z*, rel. int., %); 374 (M^+) (1) HRMS *m/z* (rel. int., %): $[M]^+$ 374.2106 (1), for $C_{22}H_{30}O_5$ (calcd. 374.2093). ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.91 (3H, s, H-12), 0.94 (3H, d, $J = 6.2$ Hz, H-15), 0.95 (3H, s, H-13), 1.23 (3H, s, H-14), 1.59, (H, ddd, $J = 12.5, 6.0, 3.0$ Hz, H-10α), 1.83 (3H, d, $J = 7.0$ Hz, H-4′), 1.89 (3H, br s, H-5′), 2.10 (H, dd, $J = 13.0, 6.0$ Hz, H-10β), 2.18 (H, s, OCC$\underline{H}_3$), 2.24 (H, d, $J = 4.4$ Hz, H-7), 2.63 (H, m, H-9β), 5,27 (H, br t, $J = 3.3$ Hz, H-11α), 5.44 (H, s, H-5β), 6.15 (H, d, $J = 6.2$ Hz, H-2), 7.60 (H, d, $J = 6.2$ Hz, H-1), 7.0 (H, dq, $J = 7.0$ Hz, H-3′). ^{13}C NMR ($CDCl_3$, 50 MHz): δ_C 211.1 (s, C-3), 173.9 (s, C-1″), 168.0 (s, C-1′), 167.8 (d, C-1), 138.0 (d, C-3′), 131.9 (d, C-2), 86.3 (d, C-5), 76.1 (d, C-11), 63.7 (s, C-8), 61.5 (d, C-7), 57.0 (s, C-4), 42.6 (s, C-6),

41.7 (t, C-10), 35.0 (d, C-9), 25.0 (q, C-12), 23.7 (q, C-13), 21.7 (q, CO$\underline{C}H_3$), 19.6 (q, C-14), 15.9 (q, C-15), 14.5 (q, C-4′) 12.1 (q, C-5′).

*11β-Acetoxy-5α-isobutyryloxysilphinen-3-one (**3**).* Oil; EI-MS (70 eV, *m/z*, rel. int.,%); 362 ((3), 320 (8), 292 (42), 291 (100), 250 (22), 231 (54), 204 (46), 161 (52), 123 (49) and 83 (82); HRMS *m/z* (rel. int.,%): $[M]^+$ 362.1918 (3), for $C_{21}H_{30}O_5$ (calcd. 362.2093). ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H, 7.55 (1H, d, $J = 5.8$ Hz, H-1), 6.10 (1H, d, $J = 5.5$ Hz, H-2), 5.31 (1H, s, H-5β), 5.21 (1H, br t, $J = 3.2$ Hz, H-11α), 2.64 (1H, qq, $J = 7.0$ Hz, H-2′), 2.59 (1H, m, H-9β), 2.19 (1H, d, $J = 4.4$ Hz, H-7α), 2.12 (3H, s, AcO), 2.06 (1H, br dd, $J = 13.6, 6.3$ Hz, H-10β), 1.56 (1H, ddd, $J = 12.0, 3.0$ Hz, H-10α), 1.24 (3H, d, $J = 7.0$ Hz, H-2′), 1.20 (3H, d, $J = 7.0$ Hz, H-3′), 1.18 (3H, s, H-14), 0.92 (3H, d, $J = 7.5$ Hz, H-15), 0.89 (3H, s, H-13), 0.86 (3H, s, H-12). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 218.3 (s, C-3), 169.9 (s, $\underline{C}OCH_3$), 167.7 (d, C-1), 165.6 (C-1′), 131.7 (d, C-2), 86.0 (d, C-5), 76.1 (d, C-11), 63, 8 (s, C-8), 61.5 (d, C-7), 56.9 (s, C-4), 42.4 (s, C-6), 41.7(t, C-10), 35.0 (d, C-9), 34.2 (d, C-2′), 25.0 (q, C-12), 23.7 (q, C-13), 21.7 (q, CO$\underline{C}H_3$), 19.8 (q, C-4), 19.1 (q, C-3′), 18.9 (q, C-4′), 15.9 (q, C-15).

*11β,5α-Diacetoxysilphinen-3-one (**6**).* Oil; EI-MS (70 eV, *m/z*, rel. int.); 334 (M^+) (18), 292 (77), 250 (32), 232 (24), 221 (31), 204 (52), 161 (61), 122 (100), 83 (33), and 82 (61). HRMS *m/z* (rel. int.,%): $[M]^+$ 334.1781 (18), for $C_{19}H_{26}O_5$ (calcd. 334.1780). ^{1}H NMR ($CDCl_3$, 200 MHz): δ_H 7.60 (1H, d, $J = 5.6$ Hz, H-1), 6.10 (1H, d, $J = 5.6$ Hz, H-2), 5.26 (1H, m, H-11α), 5.13 (1H, s, H-5β), 2.60 (1H, m, H-9β), 2.18 (1H, d, $J = 5.7$ Hz, H-7α), 2.15 and 2.12 (3H each, s, 2 × OAc), 2.00 (1H, d, $J = 6.0$ Hz, H-10β), 1.69 (1H, ddd, $J = 12.1, 3.5$ Hz, H-10α), 1.08 (3H, s, H-14), 0.96 (3H, s, H-13), 0.94 (3H, d, $J = 6.8$ Hz, H-15), 0.91 (3H, s, H-12). ^{1}H NMR ($CDCl_3$, 500 MHz): δ_C 212.9 (s, C-3), 170.1 (s, C═O), 169.8 (s, C═O), 168.7 (d, C-1), 129.4 (d, C-2), 85.8 (d, C-5), 75.0 (d, C-11), 67.3 (s, C-8), 62.5 (d, C-7), 59.9 (s, C-4), 44.9 (s, C-6), 42.8 (t, C-10), 35.4 (d, C-9), 31.3 (q, C-13), 21.7 (q, CO$\underline{C}H_3$), 20.8 (q, CO$\underline{C}H_3$), 20.5 (q, C-14), 15.4 (q, C-15), 15.2 (q, C-12).

*5α,11β-Diisobutyryloxysilphinen-3-one (**7**).* Oil; EI-MS (70 eV, *m/z*, rel. int., %); 390 (3), 320 (22), 319 (41), 250 (13), 249 (46), 232 (13), 231 (21), 204 (39), 162 (18), 161 (32), 123 (26), 122 (46), 91 (15), 82 (42), and 71 (100); HRMS *m/z* (rel. int.,%): $[M]^+$ 390.2411 (1), for $C_{23}H_{34}O_5$ (calcd. 390.2406). ^{1}H NMR ($CDCl_3$, 500 MHz) δ_H, 7.56 (1H, d, $J = 6.1$ Hz, H-1), 6.04 (1H, d, $J = 6.1$ Hz, H-2), 5.29 (1H, br t, $J = 3.0$ Hz, H-11α), 5.05 (1H, s, H-5β), 2.62 [2H, m, 2 × OCC$\underline{H}$ $(CH_3)2$] , 2.60 (1H, m, H-9β), 2.17 (1H, d, $J = 6.1$ Hz, H-7α), 2.02 (1H, dddd, $J = 13.9, 6.6, 2.7$ Hz, H-10β), 1.75 (1H, ddd, $J = 11.6, 5.0$ Hz, H-10α), 1.23 and 1.18 [3H each, $J = 7.2$ Hz, $CH(CH_3)_2$] 1.21, and 1.15 [3H each, d, $J = 6.2$ Hz, $CH(C\underline{H}_3)_2$], 1.08 (3H, s, H-14), 0.98 (3H, s, H-13), 0.90 (3H, s, H-12), 0.89 (3H, d, $J = 6.7$ Hz, H-15) ^{13}C NMR ($CDCl_3$, 50 MHz): δ_C 207.9 (s, C-3), 176.7 (s, O$\underline{C}$OR), 176.1 (s, O$\underline{C}$OR), 168.1 (d, C-1), 129.0 (d, C-2), 84.3

(d, C-5), 74.9 (d, C-11), 67.4 (s, C-8), 61.9 (d, C-7), 59.4 (s, C-4), 45.9 (s, C-6), 42.6 (d, C-9), 35.2 and 34.2 (d, OCOCH-), 31.3 (t, C-10), 20.2 (q, H-13), 19.5, 19.6 and 19.0 (4q, $(CH_3)_2$), 18.4 (q, H-12), 16.0 (q, H-14) and 15.0 (q, H-15).

*Silphinen-3,5,11-trione (**8**.)* Oil; EI-MS (70 eV, *m/z*, rel. int., %); 246 (M^+), (20), 218 (17), 203 (5),176 (6), 161 (10), 123 (11), 122 (26), 121 (62), 98 (11), 91 (18), and (100). HRMS *m/z* (rel. int.,%): $[M]^+$ 246.1248 (21), for $C_{15}H_{18}O_3$ (calcd. 246.1255). $_1$H NMR ($CDCl_3$, 500 MHz): δ_H 1.05 (3H, s, H-12), 1.08 (3H, s, H-13), 1.14 (3H, d, $J = 7$ Hz, H-15), 1.22 (3H, s, H-14), 2.29 (1H, br dd $J = 15.9$, 3.3 Hz, H-10α), 2.47 (1H, s, H-7α), 2.49 (1H, br dd $J = 15.5$, 7.8 Hz, H-10β), 6.16 (1H, d, $J = 6.1$ Hz, H-2), 7.87 (1H, d, $J = 6.1$ Hz, H-1). ^{13}C NMR ($CDCl_3$, 50 MHz): δ_C 214.0 (s, C-5 and C-11), 212.6 (s, C-3), 166.7 (d, C-1), 130.3 (s, C-2), 65.5 (s, C-8), 61.6 (s, C-4), 61.0 (d, C-7), 51.6 (s, C-6), 48.2 (d, C-9), 33.5 (t, C-10), 28.0 (q, C-13), 22.2 (q, C-14), 18.0 (q, C-15), 17.9 (q, C-12).

RESULTS AND DISCUSSION

Compounds **1–4** were previously isolated from *Cineraria geifolia*, and Compound (**1**) from *S. palmensis* (Jakupovic and Abraham, 1985; González-Coloma et al, 1995). Compound **4** has also been obtained as the partial hydrolysis product of **1** (González-Coloma et al., 1997), and is being described here as a natural metabolite of *S. palmensis* for the first time. Silphinene derivatives **5–8** were synthesized to carry out structure–activity studies.

The antifeedant effects of compounds **1–10**, picrotoxinin, and thymol were species-dependent (Table 1). The polyphagous *S. littoralis* was moderately sensitive to silphinenes **6** and **8** and insensitive to the GABA antagonists. *L. decemlineata*, a specialist of some Solanaceae species (Mitchell and Harrison, 1985; Hsiao, 1986; Sinden et al., 1988), was strongly sensitive to all the sesquiterpenes and GABA antagonists. *M. persicae* (more than 40 host-plant families) and *R. padi* (the most polyphagous among the cereal aphids) (Blackman and Eastop, 1985) were sensitive to compound **3** and thymol. *S. avenae* and *M. dirhodum*, both specialists of grasses and cereals as secondary hosts (Blackman and Eastop, 1985), were moderately sensitive to silphinenes (*S. avenae* responded to **3** and **5**; *M. dirhodum* to **3**) or picrotoxinin (*M. dirhodum*), and thymol (both). *D. noxia*, with the most restricted host-range (restricted to wheat and barley) (Blackman and Eastop, 1985), was the most sensitive aphid to silphinene derivatives (**1**, **3**, and **4**), picrotoxinin, and thymol. A similar species-dependent pattern was observed for the aphid antifeedant farnesol. Compound **3** was the only silphinene derivative that maintained its antifeedant activity among all the species tested with varying potencies according to their feeding ecologies. These results are consistent with the model suggesting that differences in taste sensitivity to deterrent compounds could account for the difference in host range (Bernays and Chapman,1994; Bernays et al., 2000). The fact that

TABLE 1. EFFECTIVE ANTIFEEDANT DOSES (EC_{50}) AND 95% CONFIDENCE LIMITS (LOWER, UPPER) OF TEST COMPOUNDS ON *S. Littoralis* L6 LARVAE, ADULT *L. Decemlineata*, AND FIVE SPECIES OF APTEROUS ADULT APHIDS

	Ec_{50} (nmol/cm^2) (95% CL)						
Compound	*S. littoralis*	*L. decemlineata*	*M. persicae*	*R. padi*	*S. avenae*	*M. dirhodum*	*D. noxia*
1	>140	0.8 (0.4,1.2)	>150	>150	>150	>150	31.4 (16.3, 60.4)
2	>200	0.17 (0.04, 0.6)	na[a]	na	na	na	>120
3	~100	0.08 (0.03,0.3)	29.3 (9.7,87.9)	~100	13.7 (6.6, 28.5)	35.8 (12.9, 99.4)	8.0 (4.8, 13.3)
4	>150	21.6 (7.4, 62.6)	>150	>150	>150	>150	38.8 (25.0, 61.1)
5	>200	4.8 (1.5, 15.3)	~100	>200	59.6 (3.6,110.4)	>200	>200
6	38.6 (12.3, 121.8)	1.0 (0.4, 2.5)	>150	>150	>150	>150	~100
7	>140	5.6 (2.3, 11.5)	>150	>150	>150	na	~140
8	42.4 (17.05, 106.0)	>100	na	na	na	na	>200
9	na	19.5 (12.2, 31.2)	na	na	na	na	47.5 (26.7, 84.5)
10	na	23.7 (11.4, 49.6)	na	na	na	na	na
Picrotoxinin	>150	27.5 (9.4, 80.1)	>100	>100	>100	53.1 (31.8, 88.6)	16.2 (6.6, 39.4)
Thymol	>300	3.6 (1.1, 12.9)	30.9 (20.8, 45.9)	23.7 (15.4, 36.6)	34.9 (26.1, 59.9)	32.0 (22.3, 45.8)	6.4 (3.6, 11.1)
Farnesol	>200	107.3 (59.9, 192.6)	67.1(54.5, 82.4)	7.9 (5.2, 11.9)	13.1 (8.1, 21.2)	3.7 (1.6, 8.5)	0.2 (0.02, 1.4)

[a] na, not enough compound available.

the insects were sensitive to either a silphinene analog, thymol, and/or picrotoxinin supports the hypothesis of a shared molecular mechanism for antifeedant taste chemoreception in divergent insect species as proposed by Mullin et al. (1997).

L. decemlineata responded to most of the silphinene analogs, as expected from their structural similarities with **1** and their possible biogenetic relationship (Marco et al., 1996), allowing for the study of their structure–activity relationships (Table 1). When compared to **1**, analogs **2** and **3** were 4 and 10 times more active, respectively, **6** was similarly active; and **7**, **9**, and **10** were 6–20 times less active.

Small structural changes resulted in drastic differences in antifeedant activity, suggesting a high molecular selectivity for silphinene derivatives on CPB taste chemoreception. The increased activity of compounds **2** and **3** versus **1** and **6** demonstrated that a small change at the C-5 substituent (from an angelate in **1** or an acetate in **6**, to a tiglate in **2** or an isobutyrate in **3**) had a great impact on the antifeedant potency. The reduction in activity found for compounds **4**, **5**, and **7** in contrast to **1–3** and **6** showed the importance of the C-11 acetate. The lack of activity of **8** (with carbonyl groups in C-5 and C-11) in contrast with **5** suggests that hydrogen-bonding at C-5 (with a hydroxyl substituent) may play a role in the antifeedant effect of these molecules. The activity of the related silphinenes **9** and **10** indicated that structurally related tricyclic sesquiterpenes are good CPB antifeedant candidates for future studies.

The GABA modulators thymol and picrotoxinin were also CPB antifeedants, with thymol being seven times more active than picrotoxinin (Table 1). Silphinenes **1–3** and **6** were more active than both antagonists; **1**, **5**, and **7** were as active as thymol; while **4**, **9**, and **10** had similar activity levels to picrotoxinin. Silphinene **1** has 3D structural features similar to those of picrotoxinin (Mullin et al., 1997). Furthermore, **1** and **4** are GABA antagonists at mammalian receptors. (Bloomquist, 2001), **4** being threefold more active than **1**, and **5** essentially inactive. These results paralleled their rootworm antifeedant action and effects on excitation of galeal chemoreceptors (Mullin et al., 1997), supporting the hypothesis of a GABA-mediated taste regulation in chrysomelid insects. However, the CPB antifeedant effects of these compounds did not correlate with their mammalian GABA antagonist effect, probably due to the different pharmacology of mammal and insect GABA receptors (see Bloomquist, 2001)

Table 2 shows the toxic and postingestive effects of the test compounds to *L. decemlineata* and *S. littoralis*, respectively. Beetle mortality significantly increased when injected with compounds **3**, **5**, **6**, **9**, and thymol, with **6** being the most toxic, but their toxicity levels did not correlate with their antifeedant action, as previously described for the toxicity of **1**, **4**, and **5** (Gonzalez-Coloma et al., 1997; Mullin et al., 1997), indicating that these compounds could act on both the peripheral and central nervous system of insects. None of the test compounds had negative effects on *S. littoralis* biomass gain or consumption, probably due to metabolic detoxification and/or species-related target differences.

TABLE 2. TP INJECTED (10 μg/INSECT, 20 INSECTS) AND ORAL (20 μg/INSECT, 20 INSECTS), TEST COMPOUNDS ON ADULT *L. decemlineata* AND *S. littoralis* L6 *Larvae*[a]

	L. decemlineata	*S. littoralis*	
Compound	Mortality at 72 hr (%)	$\triangle$B[b] (% control)	I[c] (% control)
1	0	102.31 ± 6.17	108.32 ± 5.77
2	0	84.03 ± 7.28	83.67 ± 40.80
3	40[d]	88.77 ± 4.91	93.56 ± 14.25
4	29	90.70 ± 6.17	95.00 ± 5.77
5	35[d]	99.85 ± 6.34	97.53 ± 5.92
6	63[d]	84.92 ± 3.66	117.84 ± 15.68
7	0	86.15 ± 7.23	95.20 ± 32.10
8	0	85.74 ± 8.75	81.49 ± 50.00
9	47[d]	109.86 ± 5.68	93.35 ± 2.58
10	12	na[e]	na
Picrotoxinin	21	104.75 ± 11.14	110.46 ± 11.14
Thymol	36[d]	89.85 ± 8.19	86.91 ± 36.4
P[f]		>0.05	>0.05

[a] Mortality data were corrected according to Abbott (1925).
[b] $\triangle$B = change in insect body weight (dry weight, mg).
[c] I = mg food consumed (dry weight, mg).
[d] Significantly different from the control, $P < 0.05$, contingency table analysis.
[e] na, not enough compound available.
[f] Treatment P-level, ANCOVA analysis with initial body weight as covariate.

In summary, based on the antifeedant effects of silphinenes, thymol, and picrotoxinin against a wide range of insect species, the structural similarities between silphinens and picrotoxinin and the action of these sesquiterpenes on mammalian GABA neuroreceptors suggest a shared GABA-mediated taste regulation in these insects. Therefore, the study of the molecular mode of action of this type of sesquiterpenes merits further investigation.

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OLFACTORY RESPONSES OF *Plutella xylostella* NATURAL ENEMIES TO HOST PHEROMONE, LARVAL FRASS, AND GREEN LEAF CABBAGE VOLATILES

G. V. P. REDDY,[1,2,*] J. K. HOLOPAINEN,[2,3] and A. GUERRERO[1]

[1]*Department of Biological Organic Chemistry*
Institute of Chemical and Environmental Research (CSIC)
Jordi Girona 18-26, 08034-Barcelona, Spain

[2]*Department of Ecology and Environmental Science*
University of Kuopio
FIN-70211 Kuopio, Finland

[3]*Agrifood Research Finland, Plant Protection*
FIN-31600 Jokioinen, Finland

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Abstract—The parasitoids *Trichogramma chilonis* (Hymenoptera: Trichogrammatidae) and *Cotesia plutellae* (Hymenoptera: Braconidae), and the predator *Chrysoperla carnea* (Neuroptera: Chrysopidae), are potential biological control agents for the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae). We present studies on the interactions between these bioagents and various host-associated volatiles using a Y olfactometer. *T. chilonis* was attracted to a synthetic pheromone blend (*Z*11–16:Ald, *Z*11–16:Ac, and *Z*11–16:OH in a 1:1:0.01 ratio), to *Z*11–16:Ac alone, and to a 1:1 blend of *Z*11–16:Ac and Z11–16:Ald. *C. plutellae* responded to the blend and to *Z*11–16:Ac and *Z*11–16:Ald. Male and female *C. carnea* responded to the blend and to a 1:1 blend of the major components of the pheromone, although no response was elicited by single compounds. Among the four host larval frass volatiles tested (dipropyl disulfide, dimethyl disulfide, allyl isothiocyanate, and dimethyl trisulfide), only allyl isothiocyanate elicited significant responses in the parasitoids and predator, but *C. plutellae* and both sexes of *C. carnea* did respond to all four volatiles. Among the green leaf volatiles of cabbage (*Brassica oleracea* subsp. *capitata*), only *Z*3–6:Ac elicited significant responses from *T. chilonis*, *C. plutellae*, and *C. carnea*, but *C. plutellae* also responded to *E*2–6:Ald and *Z*3–6:OH. When these volatiles were blended with the pheromone, the responses were similar to those elicited by the pheromone alone, except for *C. carnea* males, which had an increased

*To whom correspondence should be addressed. E-mail: G.Reddy@uku.fi

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response. The effect of temperature on the response of the biological agents to a mixture of the pheromone blend and *Z*3–6:Ac was also studied. *T. chilonis* was attracted at temperatures of 25–35°C, while *C. plutellae* and *C. carnea* responded optimally at 30–35°C and 20–25°C, respectively. These results indicate that the sex pheromone and larval frass volatiles from the diamondback moth, as well as volatile compounds from cabbage, may be used by these natural enemies to locate their diamondback moth host.

Key Words—Olfactory responses, *Trichogramma chilonis*, *Cotesia plutellae*, *Chrysoperla carnea*, *Plutella xylostella*, Lepidoptera: Yponomeutidae, sex pheromones, larval frass, green leaf volatiles, cabbage.

INTRODUCTION

Semiochemicals help natural enemies locate and recognize their hosts or prey (Vinson, 1985; Lewis and Martin, 1990; Vet and Dicke, 1992). Therefore, knowledge of the nature of these chemicals and their functional roles is important in the design of programs that use parasitoids and predators as biological control agents. The diamondback moth (DBM), *Plutella xylostella*, is one of the most serious pests of cruciferous crops throughout the world (Talekar and Shelton, 1993). All stages of the DBM are attacked by numerous parasitoids and predators, the former being the most widely studied. Polyphagous egg parasitoids, *Trichogramma* and *Trichogrammatoidea*, have received attention in the development of strategies to control DBM (Tabone et al., 1999); *Trichogramma chilonis* Ishii is reported to be particularly effective (Miura and Kobayashi, 1998). Larval parasitoids are the most predominant and efficient, particularly those belonging to two major genera *Diadegma* and *Cotesia* (*Apanteles*). *C. plutellae* Kurdjumov (Hymenoptera: Braconidae) is effective in lowland areas of the tropics and subtropics where it is the only larval parasitoid that can survive (Talekar and Shelton, 1993; Verkerk and Wright, 1996; Liu et al., 2000). In this context, *C. plutellae* is a good candidate for the biological control of DBM (Talekar and Shelton, 1993). Lacewings, *Chrysoperla* spp., are one of the most effective general predators of soft-bodied insects feeding on eggs and young larvae (New, 1988), *Chrysoperla carnea* being important for corn borers and aphids. This predator and *C. plutellae* have been used in an integrated pest management program to control DBM in cabbage (Reddy and Guerrero, 2000a).

We have conducted experiments to establish activity of DBM sex pheromone and larval frass volatiles, as well as green leaf volatiles (GLVs) from cabbage, on natural enemies of the pest. To our knowledge, this is the first time that such a study has been carried out. The main components of the DBM pheromone are (*Z*)-11-hexadecenal (*Z*11–16:Ald) and (*Z*)-11-hexadecenyl acetate (*Z*11–16:Ac) (Chow et al., 1977; Tamaki et al., 1977). An 8:2 to 4:6 blend of them was highly attractive to males in the field (Koshihara et al., 1978). Addition of 1%

(*Z*)-11-hexadecenol (*Z*11–16:OH) increased captures of males (Koshihara and Yamada, 1980). GLVs from cabbage, *Brassica oleracea* var. *capitata* L., are 1-hexanol, (*Z*)-3-hexen-1-ol, 1-hexen-3-ol, hexanal, (*E*)-2-hexenal, hexyl acetate, and (*Z*)-3-hexenyl acetate (Reddy and Guerrero, 2000b). The larval frass volatile of DBM is a mixture of dipropyl disulfide, dimethyl disulfide, methyl propyl disulfide, and dimethyl trisulfide (Auger et al., 1989).

METHODS AND MATERIALS

Insects. T. chilonis were reared on eggs of DBM in 250-ml beakers under a 16L:8D photoperiod, 28 ± 1°C, and 60–65% relative humidity. Adults of *T. chilonis* emerged eight days after the eggs had been parasitized. They were collected daily, transferred to glass tubes (10 cm long × 2.5 cm ID), and supplied with a 20% honey–water solution. Females used for experiments were 3-days old, mated, and without previous oviposition experience. *C. plutellae* were reared on DBM larvae as described above, following Fan and Ho (1971). Parasitoid cocoons were collected in glass vials and kept in a clean Plexiglas cage (18 × 15 × 15 cm) until emergence. Adults were provided with a 20% honey–water solution. One-day-old, mated females, without oviposition experience, were used for experiments. Eggs of *C. carnea*, collected from a cabbage field, were placed individually in plastic cups (6 cm long × 3 cm ID) and reared on DBM larvae as described above. Newly emerged adults were collected daily, fed with honey, fructose, protinex, water (1:1:1:1 and by volume), and transferred to acrylic cages (28 cm diam.) for oviposition. Three-6-day-old males and females were used for experiments.

Chemicals. Test compounds were obtained from commercial sources (Table 1). The synthetic pheromone blend was prepared by mixing *Z*11–16:Ald, *Z*11–16:Ac, and *Z*11–16:OH in 1:1:0.01 ratio. All solutions (1:100 v/v) were prepared in hexane.

Bioassays. Experiments were conducted in a Y-tube olfactometer made of transparent Plexiglas (3 cm ID; stem 10 cm, arms 8 cm; stem-arms angle 130°) with each arm connected to a glass container (250 ml) holding the odor source. Filtered air (30 ml/min for the parasitoids; 1.2 liters/min for the predator) was drawn through the olfactometer by a pump connected to a flowmeter. A vapor trail was generated with $TiCl_4$ to verify the airflow through the olfactometer. Whatman filter paper (4 × 2 cm) impregnated with 10 μl of test solution, left to dry for 5 min, and introduced into one of the containers was used as the odor source. The other container held untreated filter paper of the same size. The olfactometer was disconnected from the containers and thoroughly washed in soap and water, rinsed in 70% ethanol, and dried in an oven at 120°C after every three runs. The apparatus was rotated 180° after five runs to exclude directional bias. Each insect was allowed

TABLE 1. PURITY AND SOURCE OF COMPOUNDS USED IN STUDY

Compound	Chemical purity (≥%)	Source
Hexane	99.5	Merck KGaA
Synthetic pheromone compounds		
*Z*11–16:Ald	97	Shin-Etsu Chemical Co. Ltd
*Z*11–16:Ac	97	Shin-Etsu Chemical Co. Ltd
*Z*11–16:OH	97	Shin-Etsu Chemical Co. Ltd
Larval frass volatiles		
Dipropyl disulfide	98	Aldrich Chemical Co. Ltd
Dimethyl disulfide	99	Aldrich Chemical Co. Ltd
Allyl isothiocyanate	95	Aldrich Chemical Co. Ltd
Dimethyl trisulfide	98	Kodak
Green leaf volatiles		
(*Z*)-3-hexenyl acetate	98	Sigma Chemical Co. Ltd
(*E*)-2-hexenal	98	Aldrich Chemical Co. Ltd
(*Z*)-3-hexen-1-ol	98	Merck KGaA
Hexanal	98	Merck KGaA
1-Hexanol	99	Sigma Chemical Co. Ltd
1-Hexen-3-ol	98	Aldrich Chemical Co. Ltd
Hexyl acetate	99	Aldrich Chemical Co. Ltd

to respond for 10 min and was used only once. Insect behavior was recorded with the program The Observer (Noldus Information Technology, Wageningen, The Netherlands). Responses were considered positive when insects traveled at least 4 cm along the arm connected to test compounds. The mean percentage of time spent by parasitoids/predator in each arm of the olfactometer was recorded and the values analyzed using the Wilcoxon matched-pairs test (Statistica, Stat Soft Inc.). All experiments were conducted in daylight at $28 \pm 2^{\circ}$C and 60% relative humidity. To determine the optimum temperature for parasitoids/predator responses, tests were conducted at 15, 20, 25, 30, and 35°C using a blend of the DBM synthetic pheromone and (*Z*)-3-hexenyl acetate in 1:1 ratio.

RESULTS

Preliminary Y-olfactometer studies of *T. chilonis*, *C. plutellae*, and *C. carnea* indicated that hexane did not elicit any response from these insects. The parasitoids flew and/or walked towards the scent source, while the predator usually walked, hopped, or jumped since the olfactometer design did not allow them to fly.

Synthetic P. xylostella Pheromone. The responses of *T. chilonis*, *C. plutellae*, and *C. carnea* to pheromone blends and individual pheromone components are shown in Table 2. *T. chilonis* preferred the pheromone blend, the 1:1 blend of *Z*11–16:Ald and *Z*11–16:Ac, and *Z*11–16:Ac alone ($P \leq 0.001$) over the control,

TABLE 2. TIME SPENT IN EACH ARM OF Y OLFACTOMETER BY *Trichogramma chilonis*, *Cotesia plutellae*, AND *Chrysoperla carnea* ADULTS WITH SEX PHEROMONE COMPONENTS OF DIAMONDBACK MOTH

	Time spent in arm (%, mean ± SD)[a]							
					C. carnea			
	Female *T. chilonis*		Female *C. plutellae*		Male		Female	
Odor	Test	Control	Test	Control	Test	Control	Test	Control
Hexane	17.6 ± 15.7	20.5 ± 16.9	18.2 ± 16.2	16.5 ± 18.2	22.3 ± 14.5	20.9 ± 17.6	20.7 ± 20.4	21.8 ± 19.8
Pheromone blend[b]	46.0 ± 28.3***	22.5 ± 20.9	34.3 ± 17.2**	17.4 ± 20.7	35.2 ± 21.7**	19.9 ± 17.4	39.4 ± 24.6***	17.0 ± 15.4
Z11–16:Ald, Z11–16:Ac (1:1)	42.0 ± 22.1***	19.7 ± 17.0	36.3 ± 20.4**	20.3 ± 19.5	34.3 ± 17.8*	22.6 ± 20.7	35.2 ± 22.3**	20.7 ± 18.3
Z11–16:Ald	26.4 ± 23.5	23.3 ± 21.3	30.8 ± 16.2*	18.3 ± 16.8	19.2 ± 17.3	16.5 ± 15.4	21.4 ± 18.6	20.2 ± 17.1
Z11–16:Ac	48.0 ± 31.8***	24.2 ± 22.7	33.1 ± 16.6*	21.8 ± 19.7	21.5 ± 18.6	22.7 ± 20.7	17.5 ± 16.2	19.5 ± 17.9
Z11–16:OH	24.6 ± 21.6	20.0 ± 18.1	20.3 ± 18.9	24.6 ± 19.4	18.9 ± 16.2	21.3 ± 18.1	29.5 ± 13.6	23.0 ± 12.3

[a] Asterisks indicate means significantly different from controls (Wilcoxon matched pair test, $N = 30$): $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.
[b] Pheromone blend was a mixture of Z11–16:Ald, Z11–16:Ac, and Z11–16:OH in 1:1:0.01 ratio.

while no preference was shown for *Z*11–16:Ald or *Z*11–16:OH alone. *C. plutellae* was attracted to the complete pheromone blend, the 1:1 blend of *Z*11–16:Ald and *Z*11–16:Ac, as well as the individual components other than *Z*11–16:OH, which had no effect. Similarly, *C. carnea* males and females preferentially responded to the ternary and binary pheromone blends, but not to the individual compounds.

P. xylostella Frass Volatiles. The responses of *T. chilonis*, *C. plutellae*, and *C. carnea* to DBM frass volatiles is presented in Table 3. *T. chilonis* females responded only to allyl isothiocyanate ($P \leq 0.01$), while the other volatile compounds failed to elicit significant responses. *C. plutellae* females responded to dipropyl disulfide ($P \leq 0.001$), whereas the other frass volatiles elicited responses at $P \leq 0.01$. *C. carnea* females showed preference for allyl isothiocyanate and dimethyl trisulfide ($P \leq 0.001$). These compounds also elicited smaller responses in males ($P \leq 0.01$ with allyl isothiocyanate, $P \leq 0.05$ with dimethyl trisulfide).

Cabbage GLVs. The response of *T. chilonis*, *C. plutellae*, and *C. carnea* to cabbage green leaf volatiles is shown in Table 4. Among the seven GLVs tested, only (*Z*)-3-hexenyl acetate elicited responses from the two parasitoids and the predator. In addition, *T. chilonis* was attracted by hexyl acetate ($P \leq 0.05$), and *C. plutellae* by (*E*)-2-hexenal and (*Z*)-3-hexen-1-ol ($P \leq 0.01$ and $P \leq 0.05$). When the pheromone was blended with the individual GLVs in a 1:1 ratio, the responses of *C. plutellae* and *C. carnea* were greater than those elicited by the pheromone or the single compounds alone.

Temperature. The responses of *T. chilonis*, *C. plutellae*, and *C. carnea* to blends of the pheromone and (*Z*)-3-hexenyl acetate in 1:1 ratio at different temperatures are presented in Table 5. For *T. chilonis* and *C. plutellae*, optimum responses were obtained at 30–35°C, although at 25°C the former also responded significantly. *C. carnea* males and females were mostly attracted at 20–25°C.

DISCUSSION

Among semiochemicals, sex pheromones play a prominent role in attracting parasitoids to the host (Nordlund et al., 1983; Noldus and van Lenteren, 1985; Colazza et al., 1997). In the present study, we demonstrate for the first time that DBM sex pheromone components, individually or as synthetic blends, are attractants for the parasitoids *T. chilonis* and *C. plutellae* and the predator *C. carnea*, three of the main natural enemies of the DBM worldwide.

Trichogramma spp. utilize a variety of semiochemicals including plant synomones and host kairomones to find their oviposition host (Nordlund et al., 1985). For instance, a synthetic blend of sex pheromone components of *Heliothis zea* (*Z*7–16:Ald, *Z*9–16:Ald and 16:Ald) increased rates of egg parasitization by *T. pretiosum* in greenhouse bioassays and field plots of cotton (Lewis et al., 1982).

TABLE 3. TIME SPENT IN EACH ARM OF Y OLFACTOMETER BY *Trichogramma chilonis*, *Cotesia plutellae*, AND *Chrysoperla carnea* ADULTS WITH FRASS VOLATILES OF DIAMONDBACK MOTH

	Time spent in arm (%, mean ± SD)[a]							
					C. carnea			
	Female *T. chilonis*		Female *C. plutellae*		Male		Female	
Odor	Test	Control	Test	Control	Test	Control	Test	Control
Hexane	14.4 ± 17.3	16.8 ± 14.5	17.3 ± 15.3	19.9 ± 17.3	14.4 ± 17.3	16.2 ± 15.4	15.0 ± 19.1	18.2 ± 20.3
Dipropyl disulfide	22.6 ± 22.4	18.5 ± 16.2	38.0 ± 22.1***	16.9 ± 14.8	31.5 ± 22.6*	19.7 ± 18.0	35.4 ± 22.6**	20.0 ± 18.7
Dimethyl disulfide	25.3 ± 21.1	20.4 ± 18.3	36.5 ± 18.5**	22.4 ± 21.0	35.3 ± 16.8**	20.4 ± 23.7	30.7 ± 21.3**	13.2 ± 15.2
Allyl isothiocyanate	28.7 ± 21.3**	15.2 ± 14.4	34.5 ± 17.3**	19.6 ± 15.2	30.2 ± 18.8**	14.7 ± 16.9	37.0 ± 22.1***	19.9 ± 14.5
Dimethyl trisulfide	29.5 ± 13.6	23.0 ± 13.3	32.6 ± 23.8**	16.1 ± 15.4	36.5 ± 18.9*	24.0 ± 21.2	35.3 ± 23.4***	17.7 ± 15.9

[a]Asterisks indicate means significantly different from controls (Wilcoxon matched pair test, $N = 30$): $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

TABLE 4. TIME SPENT IN EACH ARM OF Y OLFACTOMETER BY *Trichogramma chilonis*, *Cotesis plutellae*, AND *Chrysoperla carnea* ADULTS WITH GLVS OF CABBAGE (*Brassica oleracea* SUBSP. *capitata*), WITH OR WITHOUT DIAMONDBACK MOTH SEX PHEROMONE

	Time spent in arm (%, mean ± SD)[a]							
					C. carnea			
	Female *T. chilonis*		Female *C. plutellae*		Male		Female	
Odor	Test	Control	Test	Control	Test	Control	Test	Control
Hexane	15.3 ± 13.8	16.6 ± 12.7	20.8 ± 17.4	18.2 ± 17.2	18.8 ± 15.0	16.6 ± 14.9	13.7 ± 12.6	17.5 ± 16.6
(*Z*)-3-Hexenyl acetate	41.0 ± 20.7***	21.4 ± 20.7	32.5 ± 23.8**	16.5 ± 18.0	31.8 ± 22.4**	16.4 ± 20.1	33.0 ± 20.3**	17.5 ± 15.2
(*E*)-2-Hexenal	16.8 ± 13.6	19.4 ± 15.8	30.0 ± 21.4**	15.9 ± 13.2	22.2 ± 19.3	20.2 ± 18.5	18.3 ± 16.9	20.4 ± 16.5
(*Z*)-3-Hexen-1-ol	21.0 ± 18.4	19.6 ± 16.3	30.8 ± 21.4*	22.3 ± 21.5	20.3 ± 17.4	21.2 ± 18.8	19.5 ± 16.4	18.7 ± 14.3
Hexanal	18.8 ± 17.0	18.3 ± 17.8	19.2 ± 16.3	15.9 ± 14.1	29.8 ± 14.6	24.0 ± 12.3	22.9 ± 20.7	21.2 ± 17.8
1-Hexanol	18.7 ± 16.6	22.5 ± 19.6	20.0 ± 15.8	17.6 ± 16.3	15.0 ± 19.1	18.2 ± 20.3	16.7 ± 12.9	16.1 ± 15.4
1-Hexen-3-ol	26.7 ± 19.3	22.2 ± 21.2	14.7 ± 12.6	18.4 ± 17.2	24.3 ± 22.4	16.5 ± 16.2	19.8 ± 17.3	18.6 ± 17.9
Hexyl acetate	32.3 ± 19.4*	21.5 ± 19.9	17.4 ± 14.2	15.2 ± 14.5	20.5 ± 18.3	20.2 ± 18.5	18.8 ± 16.8	20.2 ± 20.6
Synthetic pheromone (ph)	44.3 ± 23.8***	22.8 ± 18.4	33.6 ± 18.6**	17.6 ± 16.6	30.8 ± 21.4*	22.3 ± 21.5	38.5 ± 23.4**	20.0 ± 18.3
(*Z*)-3-Hexenyl acetate + ph[b]	48.4 ± 26.2***	23.8 ± 21.7	38.0 ± 22.1***	19.9 ± 14.5	45.2 ± 24.3***	22.1 ± 20.9	43.6 ± 24.4***	21.3 ± 19.1
(*E*)-2- Hexenal + ph[b]	41.3 ± 22.7***	20.3 ± 19.3	36.3 ± 23.4***	20.7 ± 16.0	34.3 ± 20.7**	20.5 ± 18.4	36.2 ± 22.5**	24.8 ± 21.4
(*Z*)-3-Hexen-1-ol + ph[b]	37.6 ± 23.4**	21.8 ± 17.4	30.6 ± 20.3**	19.2 ± 14.3	32.2 ± 19.4**	19.4 ± 16.9	34.3 ± 21.8**	22.5 ± 18.8
Hexanal + ph[b]	39.8 ± 21.7**	19.0 ± 17.3	31.1 ± 21.8**	20.6 ± 18.9	37.2 ± 22.3**	22.8 ± 19.0	30.3 ± 22.7**	19.2 ± 16.4
1-Hexanol + ph[b]	40.8 ± 20.4**	21.5 ± 20.7	33.7 ± 18.6**	17.6 ± 16.6	33.0 ± 24.8**	21.4 ± 18.8	35.6 ± 24.8**	23.5 ± 18.0
1-Hexen-3-ol + ph[b]	36.6 ± 22.4**	20.0 ± 18.3	34.2 ± 19.3**	21.8 ± 17.4	36.5 ± 18.5**	23.4 ± 21.9	32.5 ± 23.7**	18.5 ± 19.6
Hexyl acetate + ph[b]	40.2 ± 20.7***	18.7 ± 18.9	32.6 ± 20.7**	20.4 ± 18.6	33.8 ± 24.9**	20.8 ± 21.5	39.6 ± 25.9**	23.2 ± 21.9

[a]Asterisks indicate means significantly different from controls (Wilcoxon matched pair test, $N = 30$): $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.
[b]In 1:1 ratio.

TABLE 5. TIME SPENT IN EACH ARM OF Y OLFACTOMETER BY *Trichogramma chilonis*, *Cotesia plutellae*, AND *Chrysoperla carnea* ADULTS WITH BLEND OF SYNTHETIC DIAMONDBACK MOTH PHEROMONE AND (*Z*)-3-HEXENYL ACETATE IN 1:1 RATIO AT DIFFERENT TEMPERATURES

Temperature (°C)	Time spent in arm (%, mean ± SD)[a]							
					C. carnea			
	Female *T. chilonis*		Female *C. plutellae*		Male		Female	
	Test	Control	Test	Control	Test	Control	Test	Control
15	$34.3 \pm 22.8^{*}$	23.5 ± 22.8	$30.8 \pm 22.6^{*}$	21.4 ± 22.4	$32.0 \pm 18.5^{**}$	17.6 ± 15.8	$31.6 \pm 22.7^{**}$	19.5 ± 17.4
20	$37.5 \pm 23.5^{**}$	21.9 ± 20.3	$32.5 \pm 21.3^{*}$	22.7 ± 20.0	$46.2 \pm 22.7^{***}$	21.3 ± 23.4	$48.4 \pm 20.1^{***}$	24.3 ± 21.3
25	$46.2 \pm 25.5^{***}$	24.2 ± 23.0	$33.5 \pm 20.6^{**}$	17.4 ± 18.8	$45.4 \pm 23.8^{***}$	23.6 ± 20.1	$46.8 \pm 23.3^{***}$	25.6 ± 19.9
30	$48.8 \pm 26.2^{***}$	23.8 ± 22.7	$36.7 \pm 18.5^{***}$	20.9 ± 21.7	$33.5 \pm 20.2^{**}$	18.2 ± 16.2	$34.0 \pm 21.8^{**}$	17.2 ± 16.3
35	$45.3 \pm 22.4^{***}$	24.7 ± 20.6	$36.4 \pm 20.2^{***}$	19.6 ± 19.4	$30.6 \pm 18.3^{**}$	17.5 ± 18.8	$31.3 \pm 20.4^{**}$	20.5 ± 15.2

[a]Asterisks indicate means significantly different from controls (Wilcoxon matched pair test, $N = 30$): $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

These results were confirmed by Noldus (1988) in olfactometer bioassays. We have found that *T. chilonis* is attracted to the pheromone blend of the DBM and to one of the main pheromone components, *Z*11–16:Ac, alone or in combination with *Z*11–16:Ald, the other major component of the pheromone. Our results agree with those of Boo and Yang (2000), who reported that *T. chilonis* was attracted to the sex pheromone of *Helicoverpa assulta* and to a component of the pheromone blend *Z*11–16:Ac. The parasitoid was also attracted to *E*12–14:Ac, a component of the sex pheromone of *Ostrinia furnacalis* (Boo and Yang, 2000). However, contrasting results were obtained by Noldus and van Lenteren (1985), who reported that *Z*11–16:Ac is not always attractive to *Trichogramma* spp. Indeed, this chemical is also the main pheromone component of *Mamestra brassicae*, and elicited no responses in its parasitoid *T. evanescens*, in contrast to the response evoked by the sex pheromone released by calling female moths (Noldus and van Lenteren, 1985).

To our knowledge there are two reports on the response of *C. plutellae* to host plant volatiles. Bogahawatte and van Emden (1996) showed that female *C. plutellae* preferred the odor of the *Brassica* plant type on which they had developed, even over those volatiles from DBM-infested and damaged leaves of other *Brassica* plants. Potting et al. (1999) reported in wind-tunnel studies that this parasitoid uses stimuli from oilseed rape plants (*Brassica napus* cv. Falcon) in its in-flight searching behavior. An oviposition experience or contact with a host-damaged leaf prior to the bioassay significantly increased the response to these volatile cues. In our study, the DBM pheromone blend, as well as the two major components *Z*11–16:Ac and *Z*11–16:Ald, either individually or in a 1:1 blend, effectively attracted the braconid females.

We found that male and female *C. carnea* were also attracted to the DBM pheromone blend and to the pheromone blend devoid of *Z*11–16:OH. Similarly, two sex pheromone components of the pea aphid *Acyrthosiphon pisum* (Homoptera: Aphididae), (4a*S*,7*S*,7a*R*)-nepetalactone and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol, elicited significant electroantennogram (EAG) responses in both sexes of the lacewing (Zhu et al., 1999). However, female antennae were more sensitive to the lactone than to the lactol. In contrast, (*E*)-β-farnesene, a common component of the aphid alarm pheromone, elicited a 30% higher EAG response than either of the two former pheromone components, and (−)-β-caryophyllene, a potent inhibitor of the pheromone, also had an attractant effect on the predator (Flint et al., 1979).

Larval frass of DBM contains dipropyl disulfide, dimethyl disulfide, methyl propyl disulfide, dimethyl trisulfide, and allyl isothiocyanate, the three disulfides being responsible for the host searching behavior of the parasitoid *Diadromus pulchellus* (Auger et al., 1989). We tested these compounds, except the noncommercially available methyl propyl disulfide, and found that all chemicals exhibited responses from *C. plutellae* and both sexes of *C. carnea*, but not *T. chilonis*. *T. chilonis* was only attracted by allyl isothiocyanate. Notice the high attractivity

shown by the polyphagous predator *C. carnea*. This preference may be acquired during early developmental stages (Corbet, 1985), since *C. carnea* were reared on DBM larvae. We did not test predator populations reared on other hosts. Regarding frass activity on *C. plutellae*, only one previous report has been found on *Cotesia* spp: *C. marginiventris* attracted to hexane extracts of *Spodoptera frugiperda* frass and actual frass of the armyworm (Loke and Ashley, 1984).

We have previously identified several GLVs [(*Z*)-3-hexenyl acetate, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, hexanal, 1-hexanol, 1-hexen-3-ol, and hexyl acetate] from cabbage (*B. oleracea* subs. *capitata*) (Reddy and Guerrero, 2000b). These chemicals induced attractant/arresting behavior in DBM in a wind tunnel and (*Z*)-3-hexenyl acetate enhanced the attractant effect of this synthetic pheromone blend in the field. When we tested these compounds in the olfactometer, they elicited strong responses in *T. chilonis*, *C. plutellae*, and both sexes of *C. carnea*, while (*E*)-2-hexenal and (*Z*)-3-hexenol also atttracted *C. plutellae*. The attraction was greater when the GLVs were mixed with the pheromone blend in 1:1 ratio (Reddy and Guerrero, 2000b).

There are many reports on the effect of GLVs of cruciferous plants on insect pests (Visser et al., 1979; Guerin et al., 1983; Katsoyannos and Guerin, 1984; Zhang et al., 1999). However, there are few studies on the effect of GLVs on the natural enemies of these insect pests. Among these studies, volatiles emitted by sorghum (*Sorghum bicolor* L.) in the vegetative and reproductive stages arrested *T. chilonis* females, while volatiles from pigeonpea plant (*Cajanus cajan* L.) elicited no effect in the vegetative phase of the plant, and were repellent in the reproductive stage (Romeis et al., 1997). Extracts from corn leaves and the catnip *Nepeta catania* exhibited significant EAG responses in two predatory species, *Coleomegilla maculata* and *C. carnea* (Zhu et al., 1999). While many of the corn volatiles [(α-terpineol, (*Z*)-3-hexenol, (*Z*)-3-hexenal, 1-octen-3-ol, 1-hexanol, β-farnesene, 2-phenylethanol, and β-caryophyllene) attracted equal numbers of both sexes of *C. maculata* in the field, *C. carnea* adults were only allured by 2-phenylethanol (Zhu et al., 1999). In a previous but contrasting report, β-caryophyllene was found to act as an attractant of *C. carnea* in the field (Flint et al., 1979).

In the present study, the standard deviations are relatively large. This could be due to an effect of the experimental design, since the experiments were carried out at different times of the day and the responses may vary accordingly.

In summary, our results show that the parasitoids *T. chilonis* and *C. plutellae* and the predator *C. carnea* are attracted to a variety of chemical cues associated with their host, the DBM. These cues include sex pheromone components of the host, either in a blend or alone, frass volatiles, and GLVs from the host plant. The attraction of volatiles can be greatly enhanced when the GLVs are combined with the pheromone blend of the host. It is likely that the natural enemies considered utilize all these volatile compounds to locate the host.

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DEVELOPMENTAL, GUSTATORY, AND BEHAVIORAL RESPONSES OF LEAFROLLER LARVAE, *Choristoneura rosaceana*, TO TANNIC ACID AND GLUCOSE

M. PANZUTO,[1] Y. MAUFFETTE,[1] and P. J. ALBERT[2,]*

[1]*Département des Sciences Biologiques*
Université de Québec à Montréal
C. P. 8888 Succ. A., Montréal, Québec, Canada H3C 3P8

[2]*Department of Biology*
Concordia University
1455 De Maisonneuve Blvd W., Montréal, Québec, Canada H3G 1M8

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Abstract—Soluble sugars are essential nutrients generally perceived as phagostimulants to most insects studied. However, tannins are known as digestibility reducers, hence deleterious to caterpillar development, and as deterrents as well. Previous work demonstrated that larvae of the polyphagous oblique-banded leafroller, *Choristoneura rosaceana*, performed better when reared on a control + 0.5% tannic acid diet than on the standard control diet and that larvae reared on a control + 5% glucose diet had slower development and reduced survival. This study was designed to elucidate the behavioral and neurophysiological components of the larval responses to tannic acid and glucose. *C. rosaceana* larvae were reared individually from the first to the sixth instar on one of four different artificial diets: (1) control; (2) control + 5% glucose; (3) control + 0.5% tannic acid; (4) control + 5% glucose + 0.5% tannic acid. After 14 days, larvae reared on the control + 5% glucose diet had not developed past the fourth instar, whereas a considerable proportion of larvae reared on the control + 0.5% tannic acid diet had already attained the pupal stage. Insects reared on the control or the control + 5% glucose + 0.5% tannic acid diet had intermediate development, with most larvae in the fifth instar. In addition, once the mid-sixth instar was reached, the feeding preferences to 25 and 300 mM glucose, 25 mM tannic acid, and 25 mM glucose + 25 mM tannic acid over water were assessed in two-choice tests. Feeding affected preference. Control-reared insects preferred feeding on treatments containing glucose and were not deterred by tannic acid. However, larvae that had been exposed to tannic acid during their development were deterred by tannic acid and their glucose discrimination was impaired. The sensitivity to glucose was also examined from neurophysiological recordings by

*To whom correspondence should be addressed. E-mail: albert@alcor.concordia.ca

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stimulating the sugar-sensitive cell (cell 1) on the lateral styloconic sensillum of the maxillary galea with increasing concentrations of glucose (1, 10, 25, 50, 100, 200, 300, and 500 mM). We also determined whether tannic acid was phagostimulatory, since insects develop relatively quickly on a diet containing this compound, by testing 1 mM tannic acid, 1 mM tannic acid + 300 mM glucose, and 300 mM glucose on the lateral styloconic sensilla. The traces indicated that 1 mM tannic acid was not detected by any of the four chemosensory cells in these sensilla. The combination of tannic acid and glucose produced no spikes from the sugar-sensitive cell, whereas a prominent spike activity resulted with 300 mM glucose. We concluded that, although *C. rosaceana* larvae develop faster on a tannic acid diet, this compound is not a phagostimulant. The converse is true for glucose; i.e., it stimulates the sugar-sensitive cell in the lateral styloconica in a concentration-dependent fashion. Previous dietary experience changes the sensory and behavioral responses of *C. rosaceana* to glucose. Our findings imply that not all compounds that are phagostimulatory are necessarily beneficial to an insect's fitness. Therefore, developmental studies should be interpreted in conjunction with behavioral and physiological data.

Key Words—Oblique-banded leafroller, tannic acid, glucose, electrophysiology, rearing diets, feeding preference, lateral styloconic sensillum, dose–response, chemoreception, induction.

INTRODUCTION

Prior feeding experience, i.e., an insect's rearing history, can be important in modulating the selection of a particular food or simply the responsiveness to a given chemical (Ma, 1972; Yamamoto, 1974). Changes in sensitivity of certain chemoreceptors have been documented following an induction of preference to a particular food or to an allelochemical (Blaney et al., 1986). Feeny (1976), Rhoades and Cates (1976), and Swain (1979) advanced the theory that tannins are polyphenolic resistance secondary compounds that are ubiquitous in angiosperms and that have antifeedent effects on phytophagous insects. There are two types of tannins: the hydrolyzable, such as gallic acid, which are esters of sugars, usually glucose, and condensed tannins, which are polymers of flavonols (Ayres et al., 1997). It has been assumed that tannins bind to proteins, acting as nutritional protein precipitating agents, thus reducing their digestibility (Feeny, 1976; Rhoades, 1979). Zucker (1983) highlighted the fact that not every tannin can bind with every protein or vice versa. In a classical study, Feeny (1968) showed that in the winter moth larvae, *Operophtera brumata*, pupal weight and growth rate significantly decreased when fed a diet with an addition of as little as 1% tannin that had been extracted from oak leaves. In the forest tent caterpillar, *Malacosoma disstria*, an addition to the diet of 0.5% tannic acid reduced survival and growth rates, and lethal deformations were detected in the pupae (Karowe, 1989). However, Bernays et al. (1980) found that tannic acid was a feeding stimulant in various species of Acridoideae and improved growth of the tree locust, *Anacridium melanorhodon*.

The indisputable role of sugars as nutrients to lepidopteran larvae has been emphasized in various nutritional studies (Harvey, 1974; Clancy, 1992). They are also important energy sources (Friend, 1958) for insects in their later larval stages (Harvey, 1974; Panzuto and Albert, 1997). Shaw et al. (1978) hypothesized that if an insect obtains enough energy compounds (sugars) from its diet, more dietary protein will be directed toward growth. In fact, there is often a direct relationship between adult weights and rates of larval development with increasing dietary levels of some sugars (Harvey, 1974). However, this is not always the case. Clancy (1992) explored the role of sugars in conjunction with varying nitrogen levels in the western spruce budworm, *Choristoneura occidentalis*. This insect performed best when reared on diets containing 6% sucrose, an amount reflecting the lower limit observed for host foliage. Savopoulou-Soultani et al. (1994) found that $>2.3\%$ glucose levels slowed down larval development of the grape berry moth, *Lobesia botrana*, also a tortricid. The oblique-banded leafroller, *Choristoneura rosaceana* is polyphagous and feeds on a wide range of conifers (Chapman and Lienk, 1971). It also feeds on members of the Rosaceae and is notorious for its preference for apples (Carrière et al., 1995). Carrière (1992) found that Red Delicious apples (*Malus domestica*), trembling aspen (*Populus tremuloides*), wild rose (*Rosa* sp.), and snowberry (*Shepherdia canadensis*) were the preferred host species, in that order. Dauphinais (1998) demonstrated that *C. rosaceana* performed better when reared on a control + 0.5% tannic acid diet than on the standard control diet. In addition, insects reared on a control + 5% glucose diet developed more slowly and larval survivorship decreased. Thus, elevated sugar levels seemed to be detrimental to insect growth. The present study is an attempt to elucidate the behavioral and neurophysiological components of these occurrences.

All caterpillars investigated to date are known to possess gustatory receptors to sucrose and glucose (Dethier and Kuch, 1971; Schoonhoven, 1987). Sugars are perceived by a cell, and in some instances by more than one cell (Dethier, 1973; Dethier and Kuch, 1971), usually on either the medial or the lateral styloconic sensilla of the galea (Schoonhoven, 1987). Spruce budworm larvae, *Choristoneura fumiferana* (Clem.), exhibit a strong phagostimulatory response to the sugars sucrose and glucose (Albert et al., 1982; Albert and Parisella, 1988; Albert and Bauce, 1994; Panzuto and Albert, 1997). As for tannins, many have assumed that they can be perceived by contact chemoreceptors (Bennet, 1965; Chan et al., 1978; Bernays, 1981). A receptor cell responsible for the detection of these compounds has not yet been found; however, tannins do reduce the responsiveness to other compounds by acting on other cells, such as the sugar-sensitive cells in two phytophagous caterpillars (Dethier, 1982).

In the present study, we used the oblique-banded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) as a model system in an attempt to answer the following questions: (1) Do rearing conditions induce a feeding preference and/or a physiological change in responsiveness? (2) Can *C. rosaceana* detect

tannic acid with the styloconic sensilla of the galea? (3) Is glucose a phagostimulant or a deterrent? To answer these questions, behavioral and neurophysiological techniques were used to assess the response of *C. rosaceana* larvae to glucose and tannic acid when reared on four types of diet.

METHODS AND MATERIALS

Oblique-banded leafrollers, *Choristoneura rosaceana*, were obtained as egg masses from a lab colony at Agriculture et Agroalimentaire Canada located in St-Jean-sur-Richelieu, Québec, and maintained in an incubator at a temperature of 24°C, under a 16L:8D photoperiod and 60% relative humidity. Once the larvae emerged, they were transferred individually into Solo cups containing the pinto-bean-based artificial diet (Shorey and Hale, 1965) and three variations of this diet. Therefore, a total of four types of diets were used: (1) control diet, with no additions (Shorey and Hale, 1965); (2) control diet + 0.5% tannic acid; (3) control diet + 5% glucose; (4) control diet + 0.5% tannic acid + 5% glucose. The additions were achieved by measuring the total liquid volume of the preparation of the control standard diet, i.e., 5% glucose (= 67.5 g) and 0.5% tannic acid (= 6.75 g) of the final liquid volume (1350 ml). It is important to note that a 5% addition of glucose corresponds to 278 mM, whereas 0.5% tannic acid is a concentration of 2.94 mM. The insects were reared individually from the first through the sixth instars in Solo cups.

Development. A developmental assay similar to that of Dauphinais (1998) was used. Fourteen days following transfer of first instars onto the four types of diets, we inventoried the number of insects per developmental stadia for each diet. Insects reared on the control + 5% glucose diet never reached the sixth instar; hence, electrophysiology and two-choice cafeteria tests could not be performed on this group.

A 100-mg sample of ground pinto bean, a main ingredient of the control diet (Shorey and Hale, 1965), was used for the extraction of soluble sugars (Gougeon, personal communication). The sample was added to 5 ml of methanol–chloroform–water (MCW, 12:5:3, v/v/v), mixed with a vortex, and left to settle for 10 min. It was then centrifuged at 3500 rpm for 10 min; the resulting supernatant was collected, and HLPC was used to determine the type and concentration of soluble sugars. This was done to ensure that our addition of 5% glucose to the diets was the sole source of glucose present. Peaks from HPLC analysis revealed minimal amounts of the sugar alcohols mannitol and sorbitol. No glucose was found in the ground pinto bean.

Electrophysiology. Mid-sixth instar *C. rosaceana* larvae were selected from the Petri dishes and starved for 24 hr prior to an experiment. The head and the first three thoracic segments were severed from the rest of the body of a larva

and a glass microcapillary pipet containing insect saline (Schnuch and Hansen, 1989) was fitted into the opening and pushed into the walls of the hypopharynx to expose the mouthparts. Special care was taken to avoid damaging the gustatory nerve. This preparation was placed into an electrode under a compound microscope, with the animal ventral side up, to facilitate viewing and contact. A second glass micropipet was filled with a given test solution combined with 25 mM KCl and used to stimulate the lateral styloconic sensilla of the galea. Resulting action potentials were recorded for 1 sec on digital audio tape. Between each stimulation, the sensilla were allowed a 3-min rest period to avoid adaptation of the cells. Data were digitized using SAPID tools (Smith et al., 1990) and then visually analyzed by counting the number of neural impulses from the sugar, water and salt cells, which can be differentiated from one another based on their characteristic shapes and amplitudes. Direct current recordings were made to facilitate the task of distinguishing spike shapes, since the impulses were not distorted as in filtered recordings (Schnuch and Hansen, 1990).

All tests were performed on the lateral styloconic sensillum of the galea of 10–20 *C. rosaceana* larvae. This sensillum possesses a sugar-sensitive cell while the medial sensillum does not. All solutions were diluted in 25 mM KCl, an electrolyte that enables adequate electrical conductance. The solutions used to establish the dose–response curve for insects reared on the control diet were 1, 10, 25, 50, 100, 200, 300, and 500 mM glucose. The 200, 300, and 500 mM glucose data were not obtained in the same year; these were done later as a separate experiment to verify whether the curve would plateau at a concentration higher than 100 mM glucose, or whether there would be a further increase in firing frequency. The solutions for insects reared on the control diet + 0.5% tannic acid and the control diet + 0.5% tannic acid + 5% glucose were 25 and 300 mM glucose. Tannic acid at 1 mM and 1 mM tannic acid + 300 mM glucose were also tested on the lateral styloconica to verify whether tannic acid interfered with glucose perception by the sugar-sensitive cell. Preliminary tests were done to confirm that the medial styloconic sensilla did not respond to tannic acid.

Statistical analyses were done with Number Cruncher Statistical Software (J. L. Hintze, 865 East North, Kaysville, Utah 84037, USA). A one-way ANOVA ($P < 0.05$) was performed to determine whether the spike frequency of the sugar-sensitive cell in response to 100, 200, 300, and 500 mM glucose differed. A two-sample *t* test analysis ($P < 0.05$) was used to assess whether the sugar cell response differed between stimulation of 25 mM glucose and 300 mM glucose for each diet (control, control + 0.5% tannic acid, control + 0.5% tannic acid + 5% glucose). In addition, a one-way ANOVA ($P < 0.05$) verified for differences among the three diets for the responses to 25 mM and 300 mM glucose.

Behavior. A two-choice test such as that described in Albert and Bauce (1994) was used to measure feeding preferences of larvae. On the day prior to each experiment, about 10–30 mid-sixth instar larvae from each diet were isolated into

individual Solo cups and starved for 24 hr. They were then placed separately in an arena containing eight Millipore HA 0.45-μm cellulose discs (4 mm diam.). Four test discs were wetted with 15-μl aliquots of one of the test solutions (either 25 mM glucose, 300 mM glucose, or 25 mM tannic acid) and four control discs were wetted with 15 μl of distilled water. Test and control discs were placed in an alternating fashion. The discs were held in place with pins arranged in a circle over a styrofoam sheet. Each circle was covered with a 3.5-cm-diam. $\times$ 2-cm-high Solo cup. The styrofoam sheet was transferred to an incubator with the same growth conditions. The experiments lasted 12 hr. Only insects that ate $>50\%$ of the total disc area were considered for analysis. For each two-choice experiment, the percent mean consumption (MPCT) was calculated in the following manner: $MPCT = T/(T + C) \times 100$ and $MPCC = C/(T + C)$, where *MPCT* is the mean percentage consumption of the test discs (glucose or tannic acid), *MPCC* is the mean percentage consumption of the control discs (water), *T* is the proportion eaten of the test discs, and *C* is the proportion eaten of the control discs.

The solutions for the behavior experiments were 25 mM glucose vs. water, 300 mM glucose vs. water, and 25 mM tannic acid vs. water tested on insects reared on the diets (control, control + 0.5% tannic acid, and control + 0.5% tannic acid + 5% glucose). Glucose was tested at 300 mM since the addition of glucose at 5% to the diet corresponds to 278 mM. All chemicals, except for the tannic acid, were obtained from Sigma Chemical, St. Louis, Missouri, USA. Tannic acid was obtained from BDH, Toronto, Canada and contains 9% impurities.

Statistical analysis was done with Number Cruncher Statistical Software (J. L. Hintze, 865 East North, Kaysville, Utah 84037, USA). The data were analyzed using a nonparametric Mann-Whitney test ($P < 0.05$) to compare the percent mean consumption of the response of the treatment vs. water for each diet insects were reared on, since the data were not normally distributed. One-way ANOVAs ($P < 0.05$) were used to compare the means of the insects reared on the three types of diets, in response to both treatments (25 vs. 300 mM glucose). Finally, other one-way ANOVAs ($P < 0.05$) checked for differences among the treatments within each diet.

RESULTS

Development. When inventory was taken after a two-week period, *C. rosaceana* reared on the control diet presented individuals in a whole range of developmental stages but predominantly in the fifth instar (Figure 1A). Insects reared on a diet containing an addition of 5% glucose did not occur past the fourth instar and, in fact, the majority were arrested in the first instar (Figure 1B). An altogether different profile was exhibited for those insects reared on a control + 0.5% tannic acid diet: most insects had attained the pupal stage (Figure 1C).

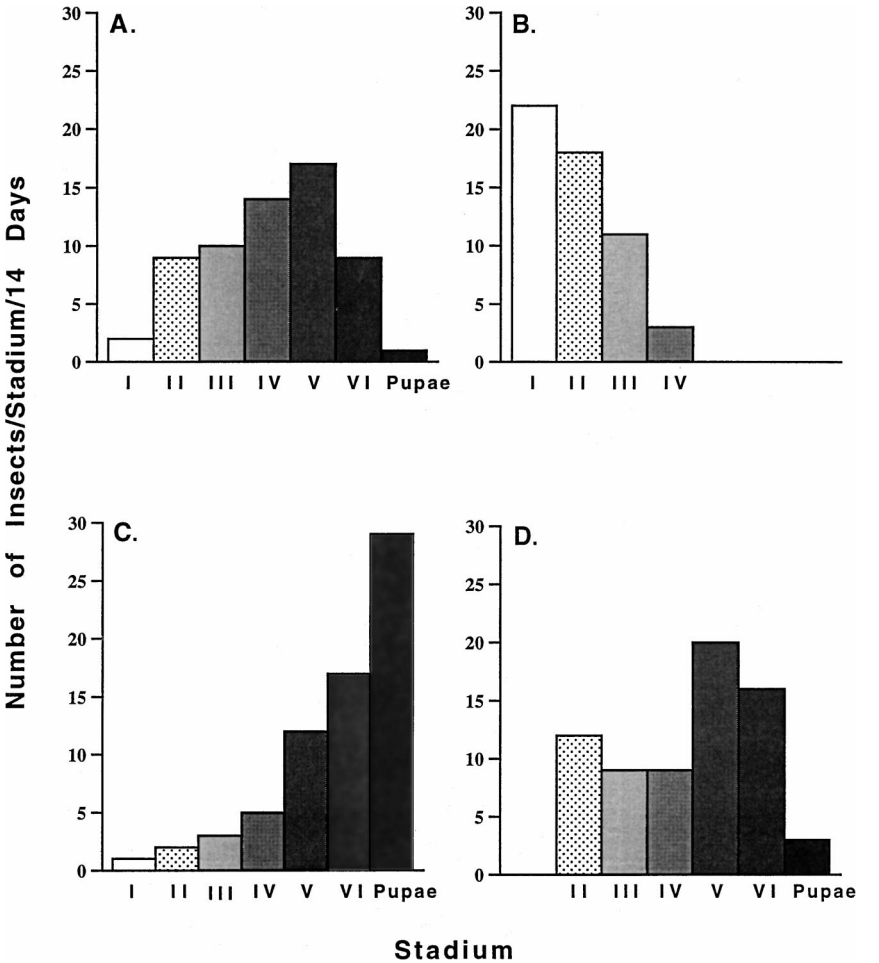


FIG. 1. Number of insects per stadium at 14 days for *C. rosaceana* reared on four different diets. A = control diet; B = control + 5% glucose; C = control + 0.5% tannic acid; D = control + 5% glucose + 0.5% tannic acid.

The combination diet (5% glucose + 0.5% tannic acid) revealed a pattern that was similar to the one seen in the case of the control diet, i.e., larvae were found in the whole range of developmental stages but mainly in the fifth instar (Figure 1D).

Electrophysiology. Three cells in the lateral styloconic sensillum responded to stimulation with the various solutions; these are identified by the characteristic shapes of their waveforms. Impulses from cell 1 (sugar cell) are monophasic,

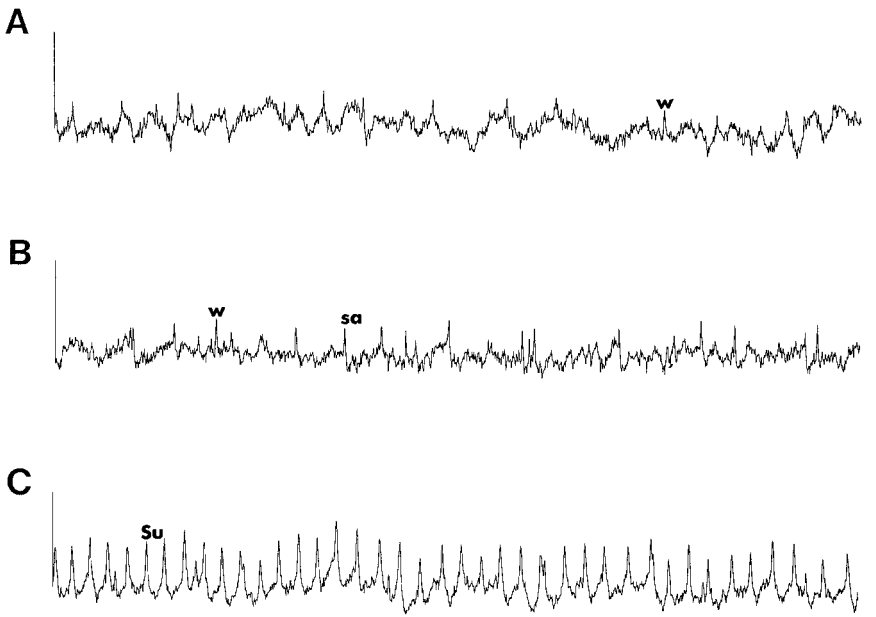


FIG. 2. Representative 1-sec traces showing the response of the lateral styloconic sensillum of sixth-instar *C. rosaceana* larvae to stimulation with: A, 1 mM tannic acid; B, 1 mM tannic acid + 300 mM glucose; and C, 300 mM glucose dissolved in 25 mM KCl. Su = sugar cell response; sa = salt cell response; w = water cell response.

slow spikes with a high amplitude (Figure 2C, Su). Cell 2 (salt cell) impulses are fast, biphasic spikes with a prominent negative flank (Figure 2B, sa). Impulses from cell 3 (water cell) are monophasic and slow, with a very low amplitude (Figure 2A, B, w).

Cell 1 (sugar cell) responded in a concentration-dependent manner to glucose (Figure 3). Maximum firing frequency was obtained at about 88 impulses/sec for animals reared on the control diet. Cell 1 showed a significant difference in response between 25 and 300 mM glucose for insects reared on the control and control + 0.5% tannic acid (Table 1). Alternatively, the control + 0.5% tannic acid + 5% glucose diet showed no significant difference between the same two glucose concentrations. The response from cell 2 (salt cell) and cell 3 (water cell) remained relatively stable at <20 impulses/sec and <5 impulses/sec, respectively (data not shown).

The one-way ANOVA comparing the cell 1 (sugar-sensitive cell) response to 25 mM glucose for *C. rosaceana* insects reared on the three diets revealed that the means were not statistically different ($F_{2,39} = 2.10$; $P = 0.136$). However, the response of this cell to 300 mM glucose showed a significant difference in mean

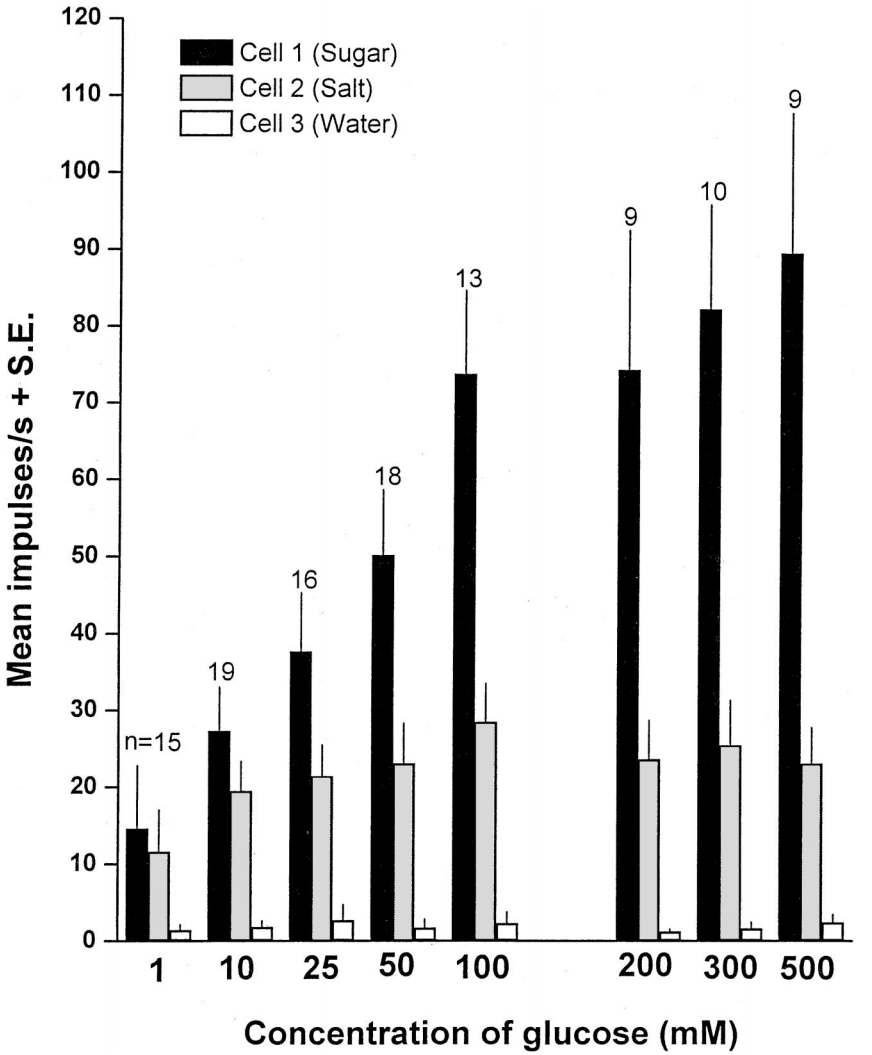


FIG. 3. Sensitivity in mean impulses/sec (+SE) of cells 1, 2, and 3 on the lateral styloconic sensillum of *C. rosaceana* larvae to stimulation with several concentrations of glucose dissolved in 25 mM KCl (N = sample size for each stimulus). Data for 200, 300, and 500 mM were collected in a separate experiment.

impulses per second depending on the type of diet ($F_{2,30} = 23.56$; $P < 0.001$). Insects reared on the control diet were significantly more sensitive to glucose stimulation (82 impulses/sec) as opposed to the responses from the other two diets which did not differ (approximately 60 impulses/sec) (Table 1).

TABLE 1. RESPONSE OF CELL 1 (SUGAR CELL) ON LATERAL STYLOCONIC SENSILLUM OF *C. rosaceana* LARVAE REARED ON DIFFERENT DIETS TO STIMULATION OF 25 AND 300 mM GLUCOSE DISSOLVED IN 25 mM KCl

Diet	Response (impulses/sec, mean ± SE)		
	25 mM glucose	300 mM glucose	*P* (*t* test)
Control	37.7 ± 7.66	82.4 ± 13.69	<0.001
Control + 0.5% tannic acid	34.4 ± 5.29	57.6 ± 7.89	0.001
Control + 0.5% tannic acid + 5% glucose	45.8 ± 13.12	63.7 ± 14.77	0.169

Stimulation of the lateral styloconic sensillum with 1 mM tannic acid in 25 mM KCl revealed traces possessing only background noise and a few cell 2 (salt-sensitive cell) and cell 3 (water-sensitive cell) spikes (Figure 2A). An addition of 300 mM glucose to the same amount of tannic acid showed a very similar response (Figure 2B). However, stimulation with 300 mM glucose (in KCl) alone resulted in a heightened activity of cell 1 (sugar cell) (Figure 2C).

Behavior. In the behavioral tests, *C. rosaceana* larvae reared on the control diet revealed a trend towards a preference for the test discs with glucose (25 or 300 mM glucose, or 25 mM glucose + 25 mM tannic acid) over water (Figure 4A). The only exception was the tannic acid vs. water test, where there was no preference. The highest percent mean consumption was obtained at ca. 75% with 300 mM glucose tested vs. water.

The diet containing the control + 0.5% tannic acid produced larvae that responded to 300 mM glucose but not to 25 mM glucose vs. water (Figure 4B). The percent mean consumption eaten for 25 mM glucose was about 55% as opposed to 78% for 300 mM glucose. In the response to tannic acid, there was a definite preference for water over 25 mM tannic acid and no difference between 25 mM glucose + 25 mM tannic acid vs. water.

The feeding response to both 25 and 300 mM glucose in control + 5% glucose + 0.5% tannic acid-reared insects shows a significant preference for glucose over water (Figure 4C). In both tests where tannic acid was tested over water (even in combination with 25 mM glucose) there was no preference for water.

DISCUSSION

Development. The results from the development study concur with prior work (Dauphinais, 1998) where it was observed that tannic acid-reared *C. rosaceana* larvae (control + 0.5% tannic acid) developed faster than those reared on control, control + 5% glucose + 0.5% tannic acid, and control + 5% glucose diets.

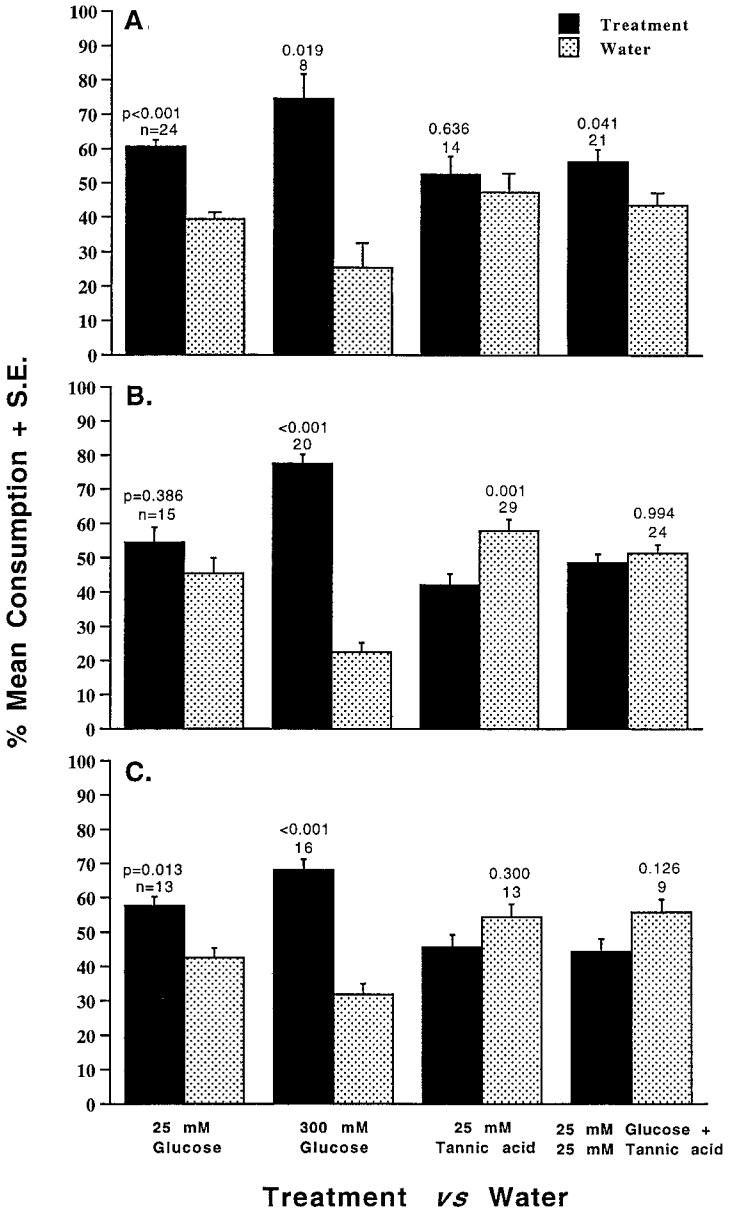


FIG. 4. Response in percent mean consumption (+SE) by *C. rosaceana* larvae reared on the control diet (**A**), control + 0.5% tannic acid diet (**B**), and the control + 0.5% tannic acid + 5% glucose diet (**C**) to a treatment vs. water. (n = sample size, p = probability value from nonparametric Mann-Whitney U test comparing the treatment *vs.* water response).

Most research on the effects of tannic acid on insects reveals its negative impact on various growth parameters including larval developmental time (Feeny, 1968, 1976; Rhoades and Cates, 1976; Rhoades, 1979; Karowe, 1989). Fundamentally, a prolonged stadium duration is not ideal since this is usually associated with increased risk of predation (Bernays, 1989) and asynchronization with the phenology of the insect's host plant (Moran, 1986). However, our finding that tannic acid supports the growth of a herbivore (Figure 1C) is not unique. The tree locust, *Anacridium melanorhodon*, makes use of the phenol gallic acid, or a derivative of it, isolated from the leaves of *Geranium endressi*, in the process of sclerotization of the cuticle. Tannic acid is hydrolyzed to gallic acid in the gut of locusts (Bernays, 1978; Bernays and Chamberlain, 1980). Furthermore, a plausible explanation to our finding that *C. rosaceana* develops more quickly on a tannic acid diet can be provided in Bernays' (1978) work wherein she describes the tree locust, *S. gregaria*, also reaps benefits from this phenolic compound. She suggests that in the hydrolysis process that occurs during the passage of tannic acid through the gut, gallic acid and glucose are released and probably utilized by the insect for its development.

As in Dauphinais's work (1998), we observe that a significant proportion of insects reared on a control + 5% glucose diet displayed an arrested development at the first stages and thus did not survive to pupation (Figure 1B). The artificial diet with 5% glucose represents a concentration of about 300 mM. However, the concentration of glucose in trees varies between 2 and 10% of dry weight, which corresponds roughly to between 10 and 50 mM in green leaves (Schoonhoven et al., 1998), suggesting that a control diet with 5% glucose addition is oversaturated with this sugar.

The developmental time for insects reared on the control + 5% glucose + 0.5% tannic acid diet was similar to that of the control diet (Figure 1A,D). Tannic acid might somehow mask the effects of glucose, maybe by complexing with the glucose molecules in the gut, therefore reducing the high concentration of glucose that was added to the diet to one where the nutritional value increases. This seems a reasonable argument since tannins are known to cross-link with nucleic acid and polysaccharides, such as cellulose and starch (Schoonhoven et al., 1998); nonetheless, we do not know whether the same process may be occurring with monosaccharides.

Electrophysiology. The response of the sugar-sensitive cell on the lateral styloconic sensilla of the galea of *C. rosaceana* is typically dose-dependent; i.e., as the concentration of glucose increases, there is a concomitant increase in the firing frequency of cell 1 (sugar-sensitive cell) (Figure 3). The sugar-sensitive cell of insects reared on the control diet exhibits a much more pronounced response to stimulation by 300 mM glucose than for insects reared on control + 0.5% tannic acid and control + 0.5% tannic acid + 5% glucose (Table 1). Compared to caterpillars reared on the control diet, caterpillars reared on the two other diets showed

a significantly lower chemosensory sensitivity to 300 mM glucose, about 80 impulses/sec as opposed to approximately 60 impulses/sec, respectively. Feeding on diets amended with tannic acid or glucose, especially at elevated concentrations, seems to alter the characteristics of chemoreceptors by decreasing their sensitivity to glucose. Sensitivity changes at the level of the peripheral chemoreceptors as a result of rearing insects on different diets are known (Blaney et al., 1986). For instance, in larvae of the tobacco hornworm, *Manduca sexta*, adding inositol or salicin to an artificial diet causes a reduction in the sensitivity of the cells responsive to these compounds (Schoonhoven, 1969).

Tannic acid did not act as a phagostimulant to *C. rosaceana* (Figure 2). In fact, addition of only 1 mM tannic acid to an otherwise stimulating 300 mM glucose solution resulted in response from only the salt-sensitive cell. Tannic acid may block the excitability of the sugar-sensitive cell, perhaps since, like other phenols, it possesses an aromatic ring (Denno and McClure, 1983), which is a rather bulky molecule when it comes in contact with sugar receptor sites. Dethier (1982) observed that tent caterpillars, *Malacosoma americanum*, do not possess a receptor that fires in response to tannic acid; however, this compound inhibited the sugar-sensitive cell in this insect. Whether it can stimulate a specific chemosensory neuron in a chemosensillum other than the lateral or medial styloconic sensilla is not known.

Behavior. We report another instance where feeding history has a subsequent effect on the behavior of an insect (Ma, 1972; Yamamoto, 1974; Städler and Hanson, 1978; Blaney et al., 1986); the feeding response of *C. rosaceana* to glucose, when presented in combination or alone, is altered due to feeding experience (Figure 4). Larvae reared on a standard control diet significantly prefer to feed on the various treatment discs containing glucose, whereas glucose discrimination is impaired in tannic acid-fed larvae where 25 mM glucose is not enough to elicit a preference. Interestingly, larvae that had not been exposed to tannic acid at any point in their development prior to the feeding tests showed a preference for discs containing tannic acid and glucose. Larvae fed a diet with an addition of 0.5% tannic acid were deterred by tannic acid. Similar results were found in Städler and Hanson's (1978) work with the tobacco hornworm, *Manduca sexta*, where they showed that the response to the deterrent was significantly reduced in larvae reared on the diet containing the deterrent. Some feedback mechanism may be involved in signaling to the insect that the choice is not nutritionally adequate. Insects are known to select a suitable host based on their internal state (Blaney et al., 1986).

Generally, consumption is reduced at very high concentrations of sugars (Ito, 1960; Schoonhoven, 1968; Chapman, 1974), which is not the case in our study. Larvae of *C. rosaceana* were stimulated to feed on glucose, even at 300 mM, which is uncommonly high considering that they do not encounter such high concentrations in the leaves of their hosts (Schoonhoven et al., 1998). As expected, the sugar-sensitive cell on the lateral styloconic sensilla responds in a

typical concentration-dependent fashion to glucose (Figure 3). However, what is remarkable in this study is that although larvae respond positively to very high concentrations of glucose, they develop poorly on a diet containing a 5% glucose addition (Figure 1B). Thus, very high concentrations were detrimental to development, as was the case in Clancy's (1992) work with the western spruce budworm, *C. occidentalis*.

In conclusion, notwithstanding the fact that tannic acid reduces instar duration, this compound is not a phagostimulant for *C. rosaceana* larvae. Quite the contrary, it may interfere with sugar binding at the receptor sites of the sugar-sensitive cell in the lateral styloconic sensilla. Glucose is highly phagostimulatory for these larvae; at high concentrations it elicits a response from the lateral styloconic sensillum of the galea even if deleterious effects are incurred in their development. Rearing conditions profoundly affect feeding preferences; 0.5% tannic acid and 5% glucose additions to the diet impair the response to sugar. Hence, both behaviorally and physiologically, tannic acid is not a phagostimulant for this insect. The sensory and feeding responses of *C. rosaceana* are definitely altered due to feeding experience. The interactions between tannic acid and glucose that we observe in our development, behavior, and electrophysiology tests suggest that we should be cautious in making general conclusions about the roles of specific compounds on the decision-making processes of feeding. The significance of these interactions may become more ecologically apparent once we obtain detailed information on the relative concentrations of tannins and glucose in the various host plants of this polyphagous species.

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IN VIVO VOLATILE EMISSIONS FROM PEANUT PLANTS INDUCED BY SIMULTANEOUS FUNGAL INFECTION AND INSECT DAMAGE

YASMIN J. CARDOZA,[1] HANS T. ALBORN,[2] and JAMES H. TUMLINSON[2,]*

[1]*Department of Entomology and Nematology*
University of Florida
Gainesville, Florida 32611-0620

[2]*Center for Medical, Agricultural and Veterinary Entomology*
United States Department of Agriculture
1700 SW 23rd Drive, Gainesville, Florida 32604

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Abstract—Peanut plants, *Arachis hypogaea*, infected with white mold, *Sclerotium rolfsii*, emit a blend of organic compounds that differs both quantitatively and qualitatively from the blend emitted from plants damaged by beet armyworm (BAW; *Spodoptera exigua*) larvae or from uninfected, undamaged plants. Attack by BAW induced release of lipoxygenase products (hexenols, hexenals, and hexenyl esters), terpenoids, and indole. The plant-derived compound methyl salicylate and the fungal-derived compound 3-octanone were found only in headspace samples from white mold infected plants. White mold-infected plants exposed to BAW damage released all the volatiles emitted by healthy plants fed on by BAW in addition to those emitted in response to white mold infection alone. When BAW larvae were given a choice of feeding on leaves from healthy or white mold-infected plants, they consumed larger quantities of the leaves from infected plants. Exposure to commercially available (*Z*)-3 hexenyl acetate, linalool, and methyl salicylate, compounds emitted by white mold-infected plants, significantly reduced the growth of the white mold in solid-media cultures. Thus, emission of these compounds by infected plants may constitute a direct defense against this pathogen.

Key Words—Peanut, plant defense, volatile induction, white mold, beet armyworm, *Spodoptera exigua*, *Sclerotium rolfsii*, *Arachis hypogaea*.

*To whom correspondence should be addressed. E-mail: jtumlinson@gainesville.usda.ufl.edu

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INTRODUCTION

Plants are attacked by a number of organisms during their life-span, including insect herbivores and disease-causing microorganisms. In response, they have evolved complex defense mechanisms to fend off their parasites. These mechanisms may be triggered by a specific organism and may be effective against present and future attacks by organisms of the same or different species. This phenomenon is known as cross-resistance and has been the subject of interest for many biologists since the 1970s (Karban and Baldwin, 1997). Another field that has received much attention, especially in the last decade, is the induction of what are now called indirect defenses (i.e., volatile emissions) in plants by feeding of herbivorous insects. Induced volatile compounds released by the plant attract natural enemies of the herbivore inflicting the damage (Dicke and Sabelis, 1988; Turlings et al., 1991).

It is clear that a thorough understanding of the plant's own defense mechanisms is required for their use against the pests that present a constant threat to our agricultural commodities. Although, in a typical field situation, plants are confronted with a wide array of antagonistic organisms, most studies on induced plant defenses have been conducted using plant–pathogen or plant–herbivore systems independent of one another. However, in recent years, many studies have suggested an interaction (or cross-talk) between the pathways involved in plant defense to pathogens and herbivorous insects. In many cases, it appears that induction of plant defenses by one type of organism interferes with plant defense against the other (Karban et al., 1987; Fidantsef et al., 1999; Stout et al., 1999; Bostock, 1999; Felton et al., 1999). Much remains to be learned about how plants defend against multiple stress factors. All studies conducted on this subject have focused on inducible direct defense compounds. It is still not known how plant indirect defenses against herbivores, consisting of volatile synomones, are influenced by the presence of phytopathogens, and how simultaneous attack of insects and phytopathogens on the plant may affect production of indirect defense substances.

In the present study, we tested the effect of peanut, *Arachis hypogaea* L. (Fabaceae), stem infection by the white mold fungus, *Sclerotium rolfsii* Sacc. (Mytosporic fungi), on the feeding preference of beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae). We also evaluated and compared the emission of volatile compounds from peanut plants under attack by the white mold fungus and by beet armyworm. The emission of volatile compounds from *S. rolfsii* fungal cultures was also evaluted. Finally, we tested the effect of synthetic forms of the commercially available compounds identified from the volatile profile emitted by fungus-infected plants on the radial growth of laboratory cultures of *S. rolfsii*.

METHODS AND MATERIALS

Fungal Culture. S. rolfsii (strain 80) was grown on potato dextrose agar (PDA) Petri plates from original cultures provided by Dr. Tim Benneman (Coastal Plain Experiment Station, University of Georgia, Tifton, Georgia). Subsequent cultures were started, under sterile conditions, in our laboratory by placing sclerotia harvested from the original culture in the center of the PDA media plates. Culture plates were kept in a biological incubator with a 14L:10D cycle and maintained at 25°C and 6–70% relative humidity. To inoculate the experimental plants, fungal culture plugs were cut out of the agar plate with a #2 cork borer (5 mm diam.).

Plant and Insect Material. Georgia Green peanut seeds were provided by Drs. Tim Brenneman and Glen Raines (Coastal Plain Experiment Station, University of Georgia, Tifton, Georgia). Seeds were sown in pairs in 3.78-liter pots (16 cm diam.) containing a 1:1 (v/v) mixture of commercially available filter sand and Metromix 300 (Scotts-Sierra Horticultural Company, Marysville, Ohio). Plants were grown in an insect-free greenhouse with natural light, under Florida summer conditions (14L:10D light cycle). The greenhouse temperature was kept at 25–30°C. After emergence, seedlings were thinned to one individual per pot. Each plant received 100 ml of a 3.38 g/liter liquid fertilizer solution [20-20-20 (N-P-K)]; Peters, W. R. Grace, Fogelsville, Pennsylvania) every two weeks starting one week after emergence. Five-week-old peanut plants with six fully developed leaves on the main stem and three fully developed leaves on each of two secondary branches were used in all experiments.

Beet armyworm eggs were obtained from the rearing facilities at the USDA-IBPMRL, Tifton, Georgia. Larvae were reared on a pinto-bean artificial diet following the methodology described by King and Leppla (1984). Insects were kept in a biological incubator with a 14L:10D cycle at 25°C. Third-instar larvae were used in all experiments.

BAW Feeding on S. rolfsii-Infected Peanuts. The feeding preference of BAW larvae on old and young leaves of healthy and fungus-damaged plants was evaluated to determine whether insects were deterred from feeding on tissues of the *S. rolfsii*-infected plants. Peanut plants were infected with the fungus by distributing four culture plugs along the first three internodal spaces of the main stem. The plugs were positioned so the fungus was in direct contact with the stem. Fungal plugs were pressed against the stem so they remained in place. Each plant was then individually covered with a 3.78-liter plastic storage bag (Ziploc DowBrands L. P., Indianapolis, Indiana) to provide adequate humidity and temperature conditions for fungal growth and colonization of the plant's stem. The plants were incubated for three days, after which time lesions approximately 1 cm long could be observed at the point of fungal contact with the stem. After this incubation period, bags were removed from the plants 24 hr before being used for the experiment. *S. rolfsii* is a

nonsystemic pathogen, and only the stems of the plants were in contact with the fungus; therefore, the leaves used for the experiments were not infected.

The second-oldest (old) or newest (young) fully developed tetrafoliate leaves on the mainstem of an infected plant were paired with their counterparts from a healthy plant by confining them within Petri dish clip-cages. The leaves (four leaflets each), still attached to each of the plants, were placed side by side within a clip-cage (Alborn et al., 1996), so the caterpillars had equal access to them. Insects were deprived of food for 6 hr before the start of the experiment to ensure immediate feeding on the plant tissues. Young peanut leaves are larger than old leaves, so six third-instar larvae were confined with the young leaves, and three larvae of the same stage were confined to the old leaves. Caterpillars were removed from the leaves after 24 hr. Leaves exposed to the feeding were carefully labeled, removed from the plants, brought into the laboratory, and photocopied to estimate feeding damage. Leaf images were scanned and imported into an imaging software program (ImagePC beta version 1, Scion Corporation, Frederick, Maryland) to estimate leaf area eaten and leaf area remaining. These measurements were used to calculate the leaf area consumed by the insects on each of the treatments. Six replicates of this experiment were set up at one time in the greenhouse. This experiment was repeated twice for a total of 18 replicates.

Volatile Collections from Fungus and BAW-Damaged Peanuts. In this experiment, plant treatments consisted of control (uninfected/undamaged), BAW damage, fungus-infection, and fungus-infection plus BAW damage. Plants were inoculated with the fungus as described in the first experiment. BAW-damaged plants were exposed to feeding by six third-instar larvae within the volatile collection chambers three days after pathogen inoculation and 12 hr before the start of the first sampling period.

The aerial portion of the plant was contained within glass sleeves that rested on Teflon bases, with an opening that closed around the plant stem (Röse et al., 1996). Purified air was pumped in at the top of the chamber at a rate of 5 liters/min. Air within each of the chambers was sampled daily, at a rate of 1 liter/min, for four days in three consecutive periods: (1) 6:00 AM–12:00 PM, (2) 12:00 PM–6:00 PM, and (3) 6:00 PM–6:00 AM. Compounds emitted were collected at the downwind end of the chambers in adsorbent traps containing 25 mg Super Q (800–100 mesh) (Alltech, Deerfield, Illinois). All volatile collections were conducted in the greenhouse where plants were grown. The experiment was set up in duplicate and repeated three times on different days for a total of six replicates.

Volatile Collection from Fungal Cultures. To determine whether any of the volatile compounds collected from infected plants were also produced by the fungus directly, we collected and analyzed volatiles from culture plugs of the white mold at different stages of development. Five 1-cm-diam. plugs were made from *S. rolfsii* culture plates at 3, 5, 8, and 16 days after inoculation. Volatiles emitted from control, noninoculated PDA plates, were also collected and analyzed. Plugs

from each treatment were separately placed in Pyrex glass odor collection chambers, and volatiles were collected using a push–pull system previously described by Turlings et al. (1991). Volatiles were adsorbed by the Super Q traps described previously. Approximately 300 ml/min of purified and humidified air was passed over the culture plugs and through the collection traps for a 2-hr period. Air samples were obtained from plugs made from three different culture plates for each of the developmental times tested.

Sample Extraction and Analysis. Compounds from individual traps in the volatile collection experiments were eluted with 170 μl dichloromethane (GC/GC-MS Solvent, B&J, AlliedSignal, Inc), and then 400 ng each of *n*-octane and nonyl acetate were added to each eluted sample as internal standards. The samples were analyzed by gas chromatography with flame ionization detection (HP5890 gas chromatograph, HP7673 auto sampler, Hewlett Packard, Palo Alto, California) equipped with a 15-m $\times$ 0.25-mm-ID, 0.25-μm film thickness DB-1 capillary column (Quadrex, New Haven, Connecticut). The splitless mode injector system was set at 220°C, the column oven was held at 40°C for 1 min after injection and then programmed at 14°C/min to 180°C. The carrier gas used was helium at a flow average velocity of 19 cm/sec. For identification of compounds, selected samples were analyzed via GC-MS (HP 6890 gas chromatograph equipped with 30-m $\times$ 0.25-mm-ID, 0.25-μm film thickness HP-5 capillary column, interfaced to a 5973 Mass Selective Detector, Hewlett Packard, Palo Alto, California) in both electron impact and chemical ionization modes. The column was held at 40°C for 1 min after injection and then programmed at 10°C/min to 180°C. The carrier gas used was helium at a flow average velocity of 30 cm/sec. Isobutane gas was used as the reagent gas for chemical ionization, and the ion source temperature was set at 250°C. Individual compounds were identified by comparing their retention times to that of commercially obtained authentic samples and by comparing their mass spectra against those available in a database from the Environmental Protection Agency/National Institute of Standards and Technology.

Effect of Volatile Compounds from S. rolfsii-Infected Peanut on Radial Growth of Fungal Cultures. The effect of vapors from synthetic forms of 3-octanone, (*Z*)-3-hexenyl acetate, linalool, and methyl salicylate (Sigma-Aldrich Chemical Company, St. Louis, Missouri) on the radial growth of *S. rolfsii* on PDA culture plates was evaluated. These compounds were selected because they are present in the blend of compounds emitted by infected peanut plants and because they were commercially available. Three levels of the compounds, 3, 10, and 30 μl, were diluted in 100 μl hexane (GC/GC-MS Solvent, B&J, AlliedSignal, Inc) and loaded on to 5 $\times$ 11-mm sleeve stoppers made of natural red rubber (Wheaton, Millville, New Jersey). The loaded stoppers were allowed to equilibrate for 24 hr before using them for the experiments. The rubber stoppers were attached to the cover of the Petri dish by means of double-sided Scotch tape to prevent direct contact and contamination of the growth media. Two *S. rolfsii* sclerotia were placed in the

center of the bottom part of the plate containing the PDA media; thus, the fungus was exposed only to the vapors of the compounds emitted from the rubber stoppers for the duration of the experiment. Rubber stoppers loaded with 100 μl hexane were used in the control plates. This experiment was repeated on different days for a total of six replicates.

Statistical Analyses. Data for BAW feeding preference were analyzed by paired *t* test (Proc MEANS; SAS Institute, 1996). Data for volatile collections and the effect of volatiles on fungal radial growth were analyzed using ANOVA (Proc GLM; SAS Institute, 1996). Significant ANOVAs were followed by Tukey's mean separation test.

RESULTS

BAW Feeding on S. rolfsii-Infected Peanuts. BAW feeding was not negatively affected by infection of *S. rolfsii* on peanut plants. To the contrary, larvae consumed significantly more of the young and old leaves from fungus-infected plants than from healthy, noninfected plants (Figure 1). Fungal infection resulted in a 1.4- and 6.4-fold increase in BAW leaf area consumption for old and young leaves, respectively.

Volatile Collections from Fungi and BAW-Damaged Peanuts. Uninfected/uninfested control plants released small amounts of volatiles compared to the other treatments. Healthy and fungus-infected peanut plants exposed to BAW feeding started releasing volatiles within 24 hr. However, plants exposed to damage by *S. rolfsii* alone did not emit volatiles until the third day of collection. Volatile emission in all plants showed a diurnal pattern with peak release during period 2 (12:00–6:00 PM). Thus, data presented for this experiment are from 12:00–6:00 PM of the third day of collection.

Noninoculated control plants (Figure 2A) released relatively small amounts of volatiles, such as myrcene, β-ocimene, linalool, and (*E*)-4,8 dimethyl-1,3,7-nonatriene compared to those released by white mold-infected or BAW-damaged peanut plants. Plants infected with the white mold fungus alone released (*Z*)-3-hexenyl acetate, linalool, and relatively large amounts of (*E*)-4,8-dimethyl-1,3,7-nonatriene (Figure 2B), which were also present in emissions from BAW-damaged plants. Additionally, the compounds methyl salicylate and 3-octanone were only present in volatile emissions from plants that were infected by the fungus (Figure 2B) and not in those of plants damaged by BAW alone (Figure 2C). Noninfected peanut plants exposed to feeding by BAW released large amounts of lipoxygenase products, monoterpenes, indole, and sesquiterpenes (Figure 2C). The emission of volatiles from peanuts in response to BAW damage was not negatively affected by infection of white mold fungus on the plant. In fact, white mold-infected peanuts damaged by BAW (Figure 2D) released all the volatiles

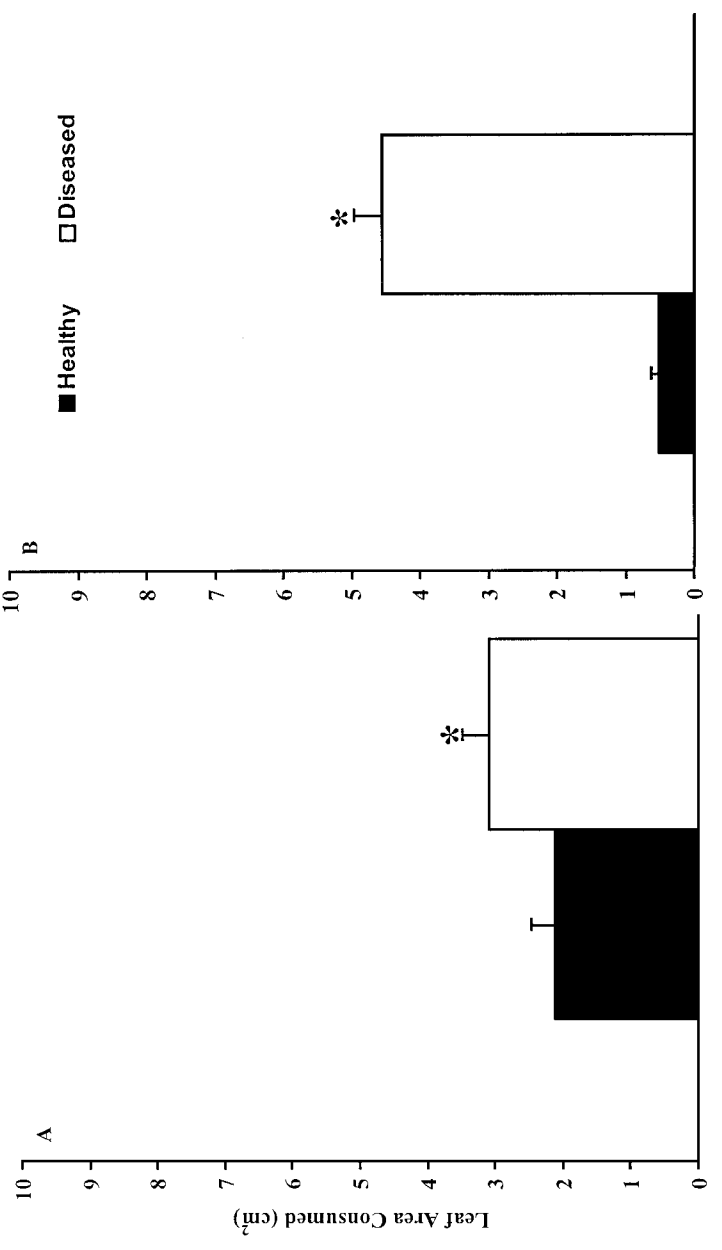


FIG. 1. Mean leaf area consumed by third-instar *Spodoptera exigua* larvae in paired-choice tests with healthy (dark bars) and white mold-infected (white bars) plants. (A) Old leaves and (B) young leaves. Error bars denote 1 SD and an asterisk denotes significant differences (paired t test, $P \leq 0.05$).

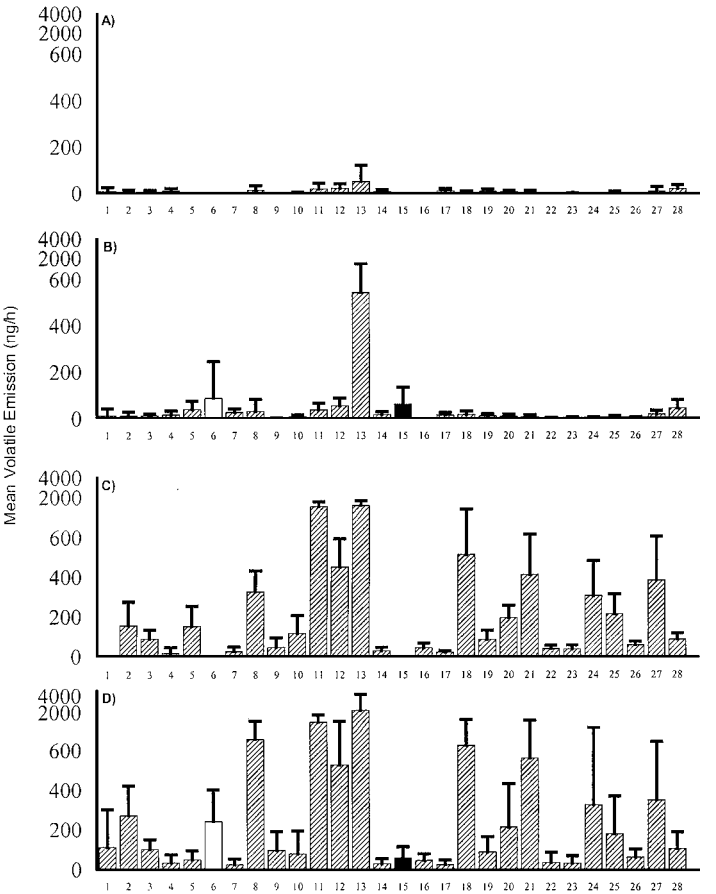


FIG. 2. Mean volatile emissions from peanut plants: (A) uninfected/undamaged, (B) white mold-infected, (C) BAW-damaged, and (D) white mold-infected + BAW-damaged. Compounds: (1) (*E*)-2-hexenal, (2) (*Z*)-3-hexen-1-ol, (3) α-pinene, (4) β-pinene, (5) 1-octen-3-ol, (6) 3-octanone, (7) (*Z*)-3-hexenyl acetate, (8) myrcene, (9) eucalyptol, (10) limonene, (11) β-ocimene, (12) linalool, (13) (*E*)-4,8-dimethyl-1,3,7-nonatriene, (14) (*Z*)-3-hexenyl isobutyrate, (15) methyl salicylate, (16) (*E*)-2-hexenyl butyrate, (17) (*Z*)-3-hexenyl butyrate, (18) indole, (19) *cis*-jasmone, (20) β-caryophyllene, (21) 2,6,-dimethyl-6-(4-methyl-3-pentenyl) bicyclo[3.1.1]hept-2-ene, (22) α-farnesene, (23) α-humulene, (24) germacrene-D, (25) β-farnesene, (26) naphthalene, (27) nerolidol, (28) (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Day 3, period 2 (12:00–6:00 PM). Bars across treatments headed with the same letter are not significantly different (Tukey's mean separation test, $P \geq 0.05$). Error bars are equivalent to 1 SD. No synthetic standards were available for compounds 21, 24, and 26; identification is based on National Institute of Standards and Technology library spectral match only.

typical of a healthy plant damaged by BAW. Furthermore, the amounts of some released compounds, such as (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, myrcene, and (*E*)-4,8-dimethyl-1,3,7-nonatriene, were higher than those emitted from noninfected plants in response to BAW feeding (Figure 2C). In addition, headspace collections from white mold/BAW-damaged plants also contained volatiles produced by plants in response to white mold infection alone (Figure 2B).

Volatile Collection from Fungal Cultures. Volatile profiles from *S. rolfsii* cultures showed the presence of one dominating volatile compound, which was identified as 3-octanone. The amounts of 3-octanone produced by the fungal culture plugs increased with age up until day 8, and ceased almost completely by day 16. Amounts of 3-octanone produced were 0.9 $\pm$ 0.4 ng/hr (mean $\pm$ SE), 1.8 $\pm$ 0.3 ng/hr, 4.3 $\pm$ 0.7 ng/hr, and 0.1 $\pm$ 0.2 ng/hr at days 3, 5, 8, and 16, respectively. This compound was recovered only from culture plates containing the fungus and not from non inoculated control PDA plates.

Effect of Selected Volatile Compounds from S. rolfsii-Infected Peanut on Radial Growth of Fungal Cultures. The growth rate of the fungus was significantly reduced by (*Z*)-3-hexenyl acetate, linalool, and methyl salicylate volatiles released from septa treated with 10 μl. Additionally, volatiles from 30 μl of linalool or methyl salicylate on a septum completely inhibited sclerotial germination and fungal growth (Figure 3). The *S. rolfsii*-produced compound 3-octanone did not prevent sclerotial germination and significantly reduced the growth of the fungus only at the highest dose of 30 μl tested.

DISCUSSION

Plant release of volatile compounds in response to attack by herbivores and the role of such compounds in attracting parasitoids of the herbivores have been studied extensively in recent years (Turlings et al., 1991, 1993; McCall et al., 1994; Loughrin et al., 1995; Röse et al., 1996, 1998; Paré and Tumlinson, 1997). In contrast, the emission of volatiles in response to pathogen invasion has been the subject of a limited number of studies (Croft et al., 1993; Doughty et al., 1996; Shualev et al., 1997; Chaudry et al., 1998; Buonaurio and Servili, 1999). All of these studies have examined the induction of plant volatile emission by either herbivore damage or pathogen infection on different plant systems or using excised plant parts. Thus, we have no clear knowledge of whether the regulation of volatile production in response to these organisms is affected in any way by the simultaneous attack of herbivores and phytopathogens on the same plant.

Data obtained from this study confirm that peanut plants release volatile compounds in response to attack by the white mold fungus. Furthermore, we present conclusive evidence that the volatile profile emitted by these plants differs qualitatively and quantitatively from profiles emitted from healthy plants and from

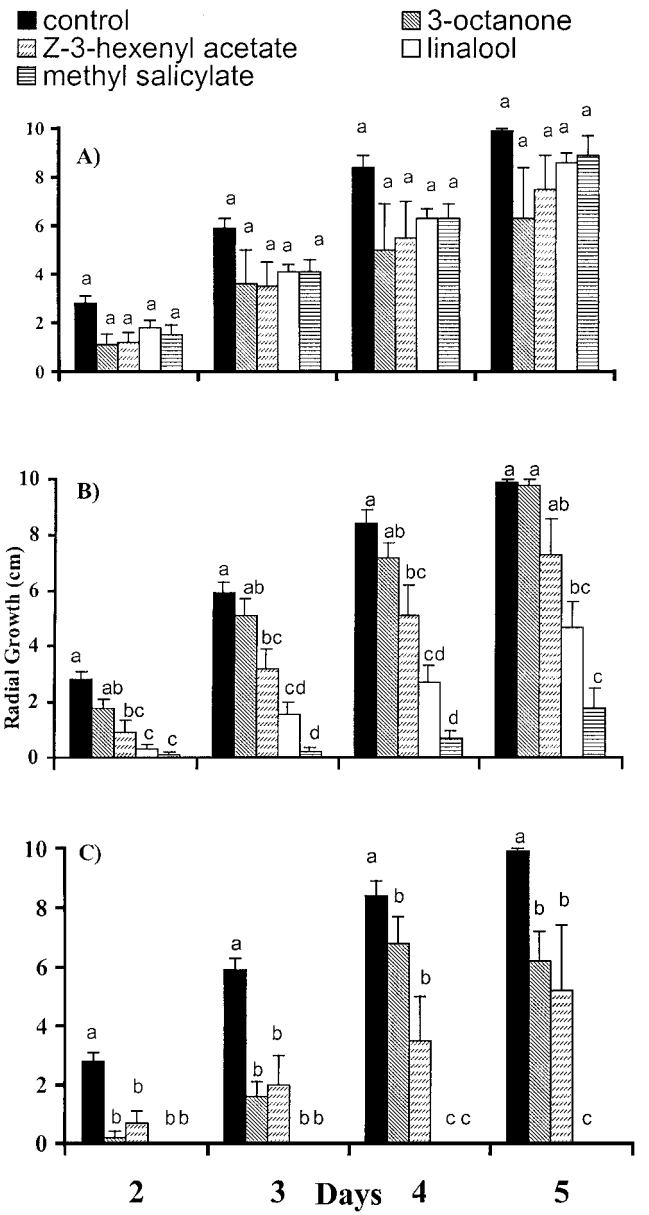


FIG. 3. Mean radial growth of *S. rolfsii* cultures exposed to volatile vapors from 3-octanone, (*Z*)-3-hexenyl acetate, linalool, and methyl salicylate: (A) 3 μl, (B) 10 μl, and (C) 30 μl of the synthetic forms of the compounds. Bars within days headed with the same letter are not significantly different (Tukey's mean separation test, $P \geq 0.05$). Error bars indicate 1 SD.

those emitted in response to BAW damage. Additionally, previous infection of the plant by the white mold fungus does not interfere with the emission of volatiles by the diseased plant in response to BAW attack; rather it seems to induce release of some compounds in relatively higher quantities.

The significant quantitative difference in the volatile profile of peanut plants attacked by either the fungus or the insect, added to the fact that emission of compounds was not suppressed when both organisms were simultaneously attacking the plant, provides a clear indication that the activation and regulation of plant biosynthetic pathways is dependent upon the type of threat perceived by the plant. Four major biosynthetic pathways are believed to be involved in the production of plant volatiles in response to damage by lepidopterous larvae (Paré and Tumlinson, 1997). The shikimic/tryptophan, mevalonate, Rohmer, and lipoxygenase pathways are all believed to be involved in the production of the different classes of volatile compounds released by plants under attack by insect herbivores (Paré and Tumlinson, 1997). The lipoxygenase (LOX) pathway, via jasmonate production, has been directly linked to direct plant defense responses to wounding and damage by herbivorous insects (Ryan, 1990; Farmer and Ryan, 1990; Farmer et al., 1992). Jasmonic acid and methyl jasmonate have also been found to induce volatile emissions similar to those resulting from herbivory in many plants (Boland et al., 1995; Dicke et al., 1999). Thus, it has been suggested that they mediate volatile production. In our study, the emission of volatiles from peanut plants previously infected with *S. rolfsii* indicates that, in this case, unlike direct defenses, volatile production is not compromised by pathogen infection. The latter may be an indication that peanut production of volatile compounds in response to insect and pathogen attack is not jasmonate-dependent. However, the precise combination and regulation of pathways activated in response to an individual or combination of stressors may vary widely among plant species. Therefore, the role of jasmonic acid and salicylic acid on the production and release of induced volatiles by plants in response to insect and pathogen attack needs to be investigated further. The dynamics of volatile emission in response to insect herbivores, phytopathogens, and their combined effect upon other plant species also merit additional attention.

Volatile compounds have been shown to affect pathogens in different ways. For example, the germination and growth of white mold are stimulated by the release of methanol and other volatile compounds emanating from moist peanut hay (Shokes et al., 1996). On the other hand, volatiles from ground-up healthy corn kernels resistant to *Aspergillus flavus* have been found to inhibit the growth and aflatoxin production in colonies of this pathogen (Zeringue et al., 1996). In cotton, however, the lipoxygenase-derived volatile (*E*)-2-hexenal inhibited, while α- and β-pinene stimulated the growth of this fungus (Zeringue and McCormick, 1989, 1990). Thus, the negative effect of (*Z*)-3-hexenyl acetate, linalool, and methyl salicylate volatiles on the growth of our *S. rolfsii* in laboratory cultures suggests that the production of these volatiles by the plant acts as a direct defense by

slowing the growth of the fungus and preventing additional sclerotial germination. However, since we were unable to quantify the headspace concentration of the compounds used, further experiments need to be conducted to confirm that the amounts are comparable to those produced by an infected plant and that in fact the growth of the fungus is hindered under natural conditions.

In the dual-choice feeding experiments, BAW preferentially fed on leaves from white mold-infected peanut plants. BAW and corn earworm, *Helicoverpa zea*, have been reported to feed more upon tomato plants that had been previously treated with benzothiadiazole–carbothionic acid *S*-methyl ester (BTH), an elicitor of systemic acquired resistance via the salicylic acid pathway (Stout et al., 1999). Tomato plants treated with BTH were found to have compromised direct defenses, based on expression of genes encoding for proteinase inhibitors (Fidantsef et al., 1999). In our study, the presence of methyl salicylate in headspace samples of white mold-infected plants indicates activity of the salicylic acid pathway in peanuts in response to *S. rolfsii* infection. Thus, the feeding preference observed in BAW towards leaves from white mold-infected peanut plants may be caused by a reduction in direct defenses of the plant due to fungal infection. Alternatively, the feeding preference of BAW for leaves from fungus-infected plants could be due to changes in the nutritional quality of the tissues caused by the pathogen infection. Different pathogen species have been found to either up- or down-regulate accumulation of photoassimilates (reviewed in Hatcher, 1995). Pathogen infections influence concentrations of compounds such as nitrogen, nonstructural carbohydrates, starch, protein, free amino acids, and amino acid composition, in their respective plant hosts (reviewed in Hatcher, 1995). Although most of the studies included in that review evaluated the effect of leaf-infecting pathogens on the suitability of plant tissue to insect herbivores, it is not inconceivable that a stem-infecting pathogen such as *S. rolfsii* can have similar consequences upon its hosts. However, this is an aspect that remains to be elucidated.

To our knowledge, this is the first in planta study in which the production of volatiles by a single host system in response to both insect herbivores and pathogens has been evaluated simultaneously. This is also the first time that the effect of previous pathogen infection on the production of plant volatiles in response to insect damage has been studied. The study of plant volatile defenses may improve our understanding of plant resistance mechanisms to disease and insect herbivores. The identification of specific pathogen- and herbivore-induced plant volatiles will greatly contribute to the development, improvement, and implementation of host-plant resistance and other control methods for insect and pathogen pests.

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EVALUATION OF SYNERGISM IN THE FEEDING DETERRENCE OF SOME FURANOCOUMARINS ON *Spodoptera littoralis*

MARIA PIA CALCAGNO,[1,*] JOSEP COLL,[2] JOAN LLORIA,[2] FRANCESCA FAINI,[3] and MIGUEL E. ALONSO-AMELOT[1]

[1]*Grupo de Química Ecológica*
Departamento de Química
Facultad de Ciencias, Universidad de Los Andes
Mérida 5101, Venezuela
mariapia@ciens.ula.ve

[2]*Departamento de Química Orgánica Biológica*
Consejo Superior de Investigaciones Científicas, CSIC
Barcelona, Spain

[3]*Departamento de Química*
Facultad de Ciencias
Universidad de Chile
Santiago, Chile

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Abstract—The phagodepression activity of five furanocoumarins (FC), bergapten, xanthotoxin, psoralen, imperatorin, and angelicin, has been studied against larvae of Egyptian cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) using a leaf-disk choice bioassay. The dose range used was 0–10 μg/cm^2 for linear furocoumarins and 0–30 μg/cm^2 for angelicin, the angular furanocoumarin. Dose–feeding deterrency activity correlations were governed by various sigmoidal functions, except in the case of imperatorin and bergapten, which had dose–response curves showing irregular traces with two maxima. All five FCs induced various degrees of phagodepression in *S. littoralis*; at 3 μg/cm^2, the relative feeding deterrence was bergapten = xanthotoxin > psoralen = imperatorin = angelicin. Structure–activity correlations indicated that a methoxy group on C-5 or C-8 enhanced the activity. Comparison of experimental feeding deterrence of binary mixtures of imperatorin with xanthotoxin, bergapten, or psoralen at various relative concentrations with the calculated additive activity of each combination indicated that a proportion of 40% or more of imperatorin may exert a greater antifeedant effect on *S. littoralis* than the sum of individual compounds. The effect of angelicin also was examined in combination

* To whom correspondence should be addressed. E-mail: mariapia@ciens.ula.ve

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with psoralen, both of which are present in *Psoralea* plants. Their mixtures yielded a clear synergistic effect in the 20–80% angelicin range (coactivity coefficient = 124–133). By contrast, the effect of angelicin on xanthotoxin was only additive. In the dose–response curves of imperatorin/bergapten combinations, synergism was found at >60% imperatorin relative concentration and 1 μg/cm^2, whereas lower proportions led to antagonism. The threshold between the two opposing effects was found to depend on the total FC concentration employed. Some natural systems contain FCs in the range of synergistic proportions recorded here and, thus, may have been produced by the host to increase its defensive effect at a lower metabolic cost.

Key Words—Furanocoumarins, synergism, *Pilocarpus goudotianus*, *Psorale glandulosa*, *Spodoptera littoralis*, feeding deterrence.

INTRODUCTION

Furanocoumarins (FC), tricyclic aromatic derivatives of the phenylpropanoid pathway, are defense metabolites of several species of plants chiefly from the Rutaceae, Apiaceae, and less commonly from the Asteraceae, Fabaceae, Pittosporaceae, Solanaceae, Thymeleaceae, Orchidaceae, and Moraceae (Dean, 1963; Murray et al., 1982). Linear and angular FCs (Berenbaum and Feeny, 1981) are toxic to plants (Fukushi, 1960; Macías et al., 1993), vertebrates and arthropods (Scott et al., 1976; Berenbaum, 1978; Ashwood-Smith et al., 1980; Berenbaum and Neal, 1985; Koenigs and Trager, 1998) and constitute an effective line of defense against insect herbivory predation (Berenbaum, 1981; Berdegué and Trumble, 1997) and possibly fungal attacks (Heath-Pagliuso et al., 1992; Oliva et al., 1999). Their role as phytoalexins has also been demonstrated (Zangerl, 1990; Masuda et al., 1998).

FCs elicit behavioral responses and physiological effects in herbivorous insects. They are repellents and feeding deterrents (Yajima et al., 1977; Yajima and Munakata, 1979; Klocke et al., 1989; Berdegué et al., 1997), and they act as phototoxins by ultraviolet radiation-promoted cycloaddition and cross-linking of DNA pyrimidines (Yajima et al., 1977; Berenbaum, 1978, 1985, 1991, 1995; Sastry et al., 1997). The combination of FC and UV light acting together against insects is consistent with the finding of these compounds in heliophilic plants. In addition, some linear FCs disrupt the detoxification capability of organisms by acting as suicide substrates for cytochrome P-450 monooxygenases, an enzyme system involved in detoxification processes in mammals (Labbé et al., 1987; Koenigs and Trager, 1998) and insects (Neal and Wu, 1994).

Selective pressure by some FCs on herbivorous insects has led to physiological adaptations that allow certain insects to consume FC-containing plants and even depend on these compounds for their reproduction and development (Nitao, 1989, 1990; Berenbaum et al., 1989; Degen et al., 1999). A good correlation was found between the diversity of FC chemistry in a given plant community and

herbivore specialization. Thus, generalists tend to exploit plants with a simpler FC composition (Berenbaum, 1981). However, these adaptations may depend on whether FCs are linear or angular (Berenbaum and Feeny, 1981).

Most FC-producing plants synthesize more than one of these compounds, and there are examples where several furano- and pyranocoumarins have been isolated from the same plant (e.g., Amaro-Luis et al., 1990). It is conceivable that the production of similarly structured materials in the same plant is an evolutionally advantageous response to a variety of environmental pressures that allows the plant to deter a wider spectrum of predators. It is also plausible that these secondary metabolites do not operate individually on the target but in concert, additively or synergistically. In the case of FCs, it has been shown that, in addition to their inherent toxicity, they may act synergistically or antagonistically when mixed with other plant secondary metabolites, FCs or otherwise. For example, mixtures of psoralen with xanthotoxin or bergapten resulted in reduced mortality of *Spodoptera exigua* reared on artificial diets mixed with these FCs and exposed to UVB radiation, suggesting an antagonistic effect (Diawara et al., 1993). In contrast, Brewer et al. (1995) found that mixtures of similar composition, but at lower combined concentration where psoralen was the minor component, caused greater mortality in *Spodoptera exigua* than the sum of individual FCs, suggesting a synergistic effect of psoralen. These results suggest that both the concentration of the mixture and the proportion of its individual components may affect the response of *S. exigua* to FCs. Additionally, synergism has been observed in mixtures of xanthotoxin with nonfuranocoumarins myristicin, safrole, and fagaramide (Berenbaum and Neal, 1985; Neal, 1989).

Feeding deterrence tests with *Spodoptera* spp. by FCs have never been performed with pairs of these compounds. Only mixtures of bergapten, xanthotoxin, and psoralen in amounts naturally found in petioles and leaves of *Apium graveolens* have been examined, but no systematic variation of their concentration was investigated. Although Berdegué et al. (1997) found apparent synergism, the contribution of each individual FC to the observed synergistic effect cannot be surmised from their data. If these and other FCs are to be deemed defense metabolites against generalist herbivores, it would be desirable to observe their effect in general mixtures of varying concentration.

Pilocarpus goudotianus (Rutaceae) is a rich source of a variety of coumarins (Amaro-Luis et al., 1990). Linear and angular pyranocoumarins and dihydrofuranocoumarins, as well as linear furanocoumarins, have been identified in ethanolic extracts of leaves in quantities that may cause toxicity in herbivores. *P. goudotianus* may rely on light-activated defenses because it grows in xerophytic areas of northwestern Venezuela and northern Colombia.

Spodoptera littoralis, a generalist herbivore is likely affected by FCs in the diet and might be sensitive to possible synergistic antifeedant effects. Thus, *Spodoptera* is a useful model to examine synergism among *P. goudotianus* furanocoumarins in reference to feeding deterrence. In particular, linear FCs (xanthotoxin, bergapten,

and imperatorin) were selected here based not only on being the most abundant FCs in *P. goudotianus*, but also because they are the most active feeding deterrents against *S. littoralis* (Calcagno, 1995). This activity was higher in chromatographic fractions that also contained imperatorin in addition to other FCs. *Psoralea*, plants contain a combination of linear and angular FCs whose possible synergistic interaction also needed to be examined. Thus, we set out to investigate the synergistic interactions of imperatorin and the other FCs in *P. goudotianus* as well as the linear–angular combinations in *Psoralea glandulosa*. Although chemical synergism in plants may result from the contribution of several compounds, methodological clarity requires that such effects should be investigated first by pairs of compounds.

METHODS AND MATERIALS

Compounds. The FC structures are shown in Figure 1. Angelicin and psoralen were isolated from *Psoralea glandulosa* collected from Cuesta La Dormida, Chile, in March 1994 (end of southern summer) according to an established procedure (Labbé et al., 1996). The three linear FCs bergapten, xanthotoxin, and imperatorin were obtained by hexane extraction and silica gel chromatography of aerial parts of several mature individuals of *Pilocarpus goudotianus*, collected in the deciduous dry forest of Curarigua, Lara State, in west-central Venezuela in May 1993 (end of dry season). Plant parts were transported to the laboratory in a cooler within 12 hr, air dried at 28–30°C for several days in the dark, and stored in paper bags at room temperature until used. This material was milled to fine particles just before solvent extraction. The antifeedant activity against *S. littoralis* of hexane,

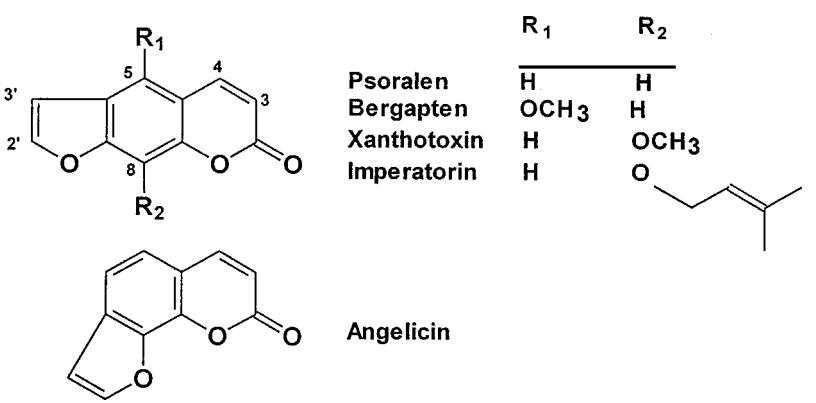


FIG. 1. Chemical structures of FCs examined in this study.

dichloromethane, and ethyl acetate extracts of this plant, which were obtained at room temperature using an ultrasound bath, were tested as described below. Only the hexane extract was found to contain the antifeedant activity. This extract (3×1 liter) of *P. goudotianus* dry leaves (200 g) was prepared by sonication at room temperature. The filtered solution was partitioned with methanol–water 9:1 (3×600 ml). After removing the methanol in a rotary evaporator at room temperature with protection from ambient light, the aqueous layer was extracted again with hexane. The crude residue (0.85 g) remaining after desiccation with anhydrous sodium sulfate, filtration, and evaporation was chromatographed through silica gel (30 g) and eluted with a hexane–dichloromethane gradient away from direct light. The fractionation was followed by antifeedant tests on larvae of *S. littoralis* (see below). The active fractions were gathered (0.34 g) and chromatographed again on the same stationary phase (15 g) using a hexane–ethyl acetate gradient. The final purification was performed using thick-layer chromatography on silica gel employing hexane–*t*-butyl methyl ether (1:2) and hexane–chloroform–methanol (8:4:0.3), and yielded three compounds. ^{1}H and ^{13}C NMR analysis showed these to be imperatorin (0.015%; mp 100–102°C, lit 102–103°C; Harkar et al., 1984), bergapten (0.009%; mp 190–193°C, lit 193–194°C; Yajima et al., 1977) and xanthotoxin (0.022%; mp 152–154°C, lit 151–152°C; Yajima et al., 1977). Upon exhaustion of the plant stock, bergapten and psoralen were purchased from Sigma Chemical Co. (St. Louis, Mo) and angelicin and imperatorin from Extrasynthese:HPLC (Genay, France) with bioactivities identical to the naturally derived compounds determined by parallel antifeedant bioassays. A stock acetone or acetone–chloroform solution, depending on compound solubility, of 1 mg/ml of each linear FC and 3 mg/ml of angelicin was prepared, from which aliquots were drawn to obtain the working concentrations, such that 10 μl of solution would contain 1–10 μg for linear FCs and 2–30 μg for angelicin. For binary mixtures, the total FC concentration was kept constant and bioassays were run employing various total concentrations in the range of 1–5 μg/cm^2 leaf disk.

Insects. Newly ecdysed fifth-instar larvae from laboratory cultures of *S. littoralis* were used for the antifeedant assays. Insects were reared on standard artificial diet (Poitout and Bues, 1974) at 25 ± 2°C, 60–70% relative humidity, 18 hr photophase, and maintained for several (>50) generations with eventual out-crossings in the insectary of CSIC-Barcelona, Spain.

Two-Way Feeding Bioassay. These were performed using the leaf disk method of Bellés et al. (1985) employing lettuce (*Lactuca sativa*) as feeding substrate at 30 ± 0.5°C in the dark for the length of the feeding period. Areas devoured by the larvae were determined by overlapping the disks on millimeter-squared paper. The feeding coefficient, FR_{50}, was calculated from the ratio of treated and control disk areas at the time 50% of the control disks had been consumed. FR_{50} varies from 0, i.e., no consumption of treated disks, to $FR_{50} > 1$ where acceptance of the treated disk indicates a net phagostimulant effect. Coactivity coefficients (CA) were used

to estimate synergism where CA is defined as:

$$CA = [(1 - FR_{50})_e/(1 - FR_{50})_c] \times 100$$

where $(1 - FR_{50})_e$ is the experimental feeding deterrence index and $(1 - FR_{50})_c$ is the calculated feeding deterrence index.

Mathematical Simulations and Statistical Methods. Computational calculations were performed with a Pentium III 500-MHz computer running Microsoft Excel 98 spreadsheet and Microcalc Origin v. 5.0 (Microcalc Software Inc., One Roundhouse Plaza, Northampton, Massachussetts) for plotting, curve fittings, and simulations. Tukey ($\alpha = 0.05$) tests for comparison of means were executed with SX v. 4.5 statistical package (Analytical Software, St. Paul, Minnesota)

RESULTS AND DISCUSSION

Activity of Individual FCs (Table 1). The phagodepression activity of FCs on *S. littoralis* might be expected to follow a linear dose–response curve. This is true for some FCs, particularly in the lower range of concentrations tested. For instance, dose–response correlations of psoralen and xanthotoxin in the range of 0–10 and 0–3 μg/disk, respectively, may be adjusted to a linear regression

TABLE 1. PHAGODEPRESSION ACTIVITY $(1 - FR_{50})$ OF INDIVIDUAL FCS IN TWO-WAY FEEDING BIOASSAY ON *S. littoralis*[a]

Dose (μg/cm^2)	Bergapten	Xanthotoxin	Psoralen	Imperatorin	Angelicin
0.25	−0.03 ± 0.05				
0.50	0.51 ± 0.08				
0.75	0.55 ± 0.07				
1.00	0.63 ± 0.07 a	0.28 ± 0.13 b	0.12 ± 0.08 b	0.21 ± 0.06 b	
1.50	0.64 ± 0.04 c	0.45 ± 0.10 cd	0.09 ± 0.11 e	0.25 ± 0.07 de	
2.00	0.71 ± 0.05 f	0.56 ± 0.10 fg	0.22 ± 0.09 h	0.29 ± 0.15 gh	0.20 ± 0.12 h
2.50	0.79 ± 0.03 i	0.67 ± 0.06 i	0.29 ± 0.07 j	0.27 ± 0.09 j	
3.00	0.83 ± 0.04 k	0.70 ± 0.08 k	0.34 ± 0.10 l	0.27 ± 0.07 l	
3.90				0.67 ± 0.07	
4.00	0.86 ± 0.03 m	0.77 ± 0.07 mn	0.52 ± 0.10 op	0.62 ± 0.03 no	0.32 ± 0.05 p
5.00		0.83 ± 0.03 q	0.56 ± 0.06 r	0.64 ± 0.05 qr	0.32 ± 0.09 s
6.00					0.36 ± 0.08
8.00		0.89 ± 0.03			0.55 ± 0.09
10.0	0.89 ± 0.03 tu	0.96 ± 0.01 t	0.89 ± 0.02 tu	0.66 ± 0.09 uv	0.56 ± 0.07 v
20.0					0.68 ± 0.03
30.0					0.77 ± 0.08

[a] Values are mean ± SEM ($N = 6$). Values with the same letter do not differ significantly ($\alpha = 0.05$, Tukey). Comparisons are between rows: different compounds at the same concentration.

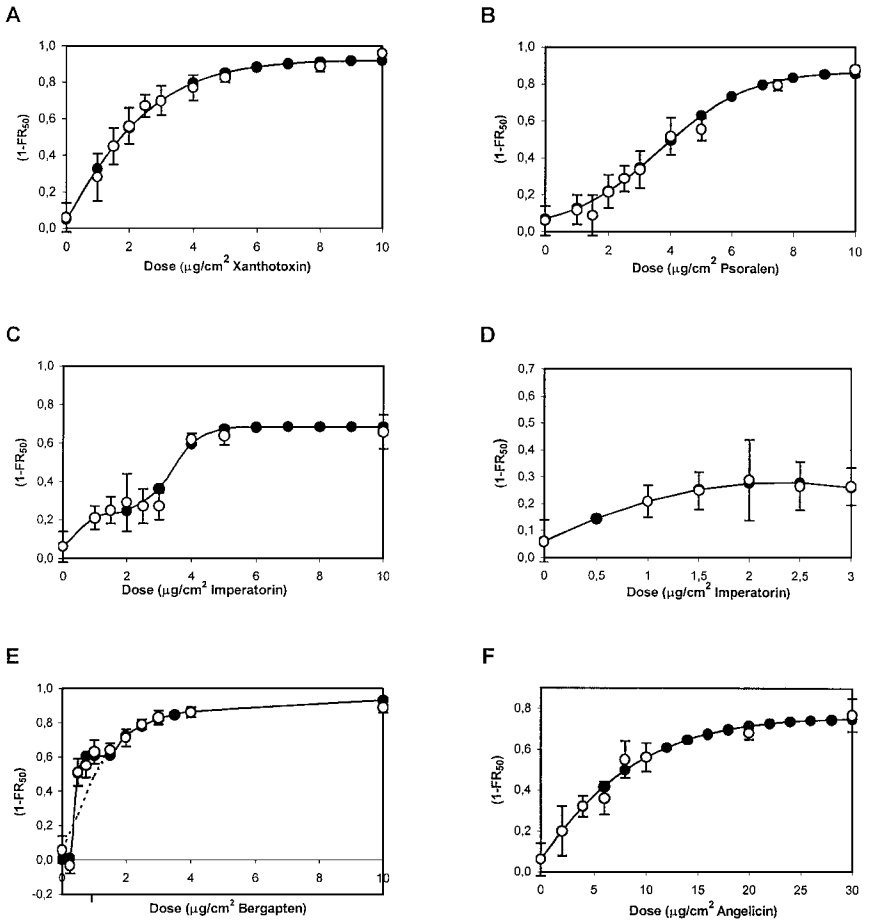


FIG. 2. Feeding deterrence dose–response plots and curve fitting models of individual furanocoumarins. (A): Xanthotoxin. (B): Psoralen. (C) and (D): Imperatorin. (E): Bergapten. (F): Angelicin. [(○) experimental; (●) calculated]. The value zero concentration is based on a control involving the insects being exposed to two sets of untreated disks. For comments and equations describing odd-shaped curves see text. Bars represent SEM.

(Figure 2). However, a better fit is obtained by representing the data as a sigmoid curve (Figure 2, Table 2). Recently, we showed that small variations in the shape of the dose–response curves may cause profound effects on the curves representing the sum of the activities of individual compounds (Alonso-Amelot and Calcagno, 2000). Therefore, computational curve fittings of dose–response correlations of individual FCs were obtained after equation and parameter choices, such that the

TABLE 2. COMPARISON OF SIGMOIDAL CORRELATIONS AND LINEAR REGRESSION OF DOSE/$(1 - FR_{50})$ ASSOCIATIONS IN XANTHOTOXIN AND PSORALEN IN THE APPARENTLY MORE LINEAR SECTION OF THE CURVES

Dose-deterrence activity correlation function		SUM |dif|[a]	
		Xanthotoxin[b]	Psoralen[c]
Boltzman sigmoidal growth	$\chi^2 = 0.0010$	0.46	
S-logistic sigmoidal growth	$\chi^2 = 0.0035$		1.34
Linear regression	$r^2 = 0.9777$	0.62	
	$r^2 = 0.9571$		1.86

[a] SUM $|\text{dif}| = \sum |[(1 - FR_{50})_e - (1 - FR_{50})_c]/(1 - FR_{50})_e|$, where $(1 - FR_{50})_e$ is the experimental feeding deterrence index and $(1 - FR_{50})_c$ is the calculated feeding deterrence index.
[b] Dosage: 0–3 $\mu g/cm^2$.
[c] Dosage: 0–10 $\mu g/cm^2$.

minimum deviation from the calculated and experimental points could be obtained. χ^2 and the sum of the module of differences between calculated and experimental values (equation 1) were used as criteria for the best fit. FR_{50} could not be employed as such because the greater the phagodepression, the lower the FR_{50}. Instead, $[1 - FR_{50}]$ inverted the scale and, thus, became amenable to additive calculations.

$$\text{SUM } |\text{dif}| = \Sigma|[(1 - FR_{50})_e - (1 - FR_{50})_c]/(1 - FR_{50})_e| \qquad (1)$$

All five of the FCs examined induced various degrees of phagodepression in *S. littoralis* (Table 1). Bergapten was the most active compound at lower dosages. Xanthotoxin, however, showed a steeper activity increment with concentration than psoralen, imperatorin, and angelicin, and reached a level of activity statistically undistinguishable from that of bergapten above 1.5 μg/disk. From 1.5 to 3.0 μg/disk, the relative feeding deterrence became bergapten = xanthotoxin > psoralen = imperatorin = angelicin. All compounds showed nonlinear dose–activity regressions, each one governed by a different equation (Table 3). The relative effect of substituents on the tricyclic FC system could be discerned by comparing their molecular structures (Figure 1). A methoxy group either on C-5 (bergapten) or C-8 (xanthotoxin) enhanced the activity relative to the unsubstituted system (psoralen) or a C-8 isoprenoxy group (imperatorin). This trend is similar to that reported by Muckensturn et al. (1981), except that we record comparable activities for psoralen and imperatorin.

Figure 2 also shows that dose–response curves in terms of $[1 - FR_{50}]$ progress towards asymptotic maxima. This suggests that the behavioral response of *S. littoralis* becomes saturated above a given threshold of FC concentrations. Beyond this limit, antifeedant tests become concentration-independent and, hence, experimentally useless.

TABLE 3. PARAMETERS OF BEST COMPUTATIONAL CURVE FITTING OF DOSE–RESPONSE PHAGODEPRESSION ACTIVITY CORRELATIONS OF INDIVIDUAL FUROCUMARINS ON *S. littoralis*

	Bergapten	Xanthotoxin	Psoralen	Imperatorin	Angelicin
Equation[a]	2	3	4	5	3
Parameter					
A1	0.059	−1.072		−0.280	−1.702
A2	0.954	0.926		0.686	0.763
Xo	1.047	−0.403			−6.199
P	1.637				
dx		1.661			6.767
A			0.883		
Xc			3.623		
K			0.675		
F				0.468	
Log X1				3.389	
Log X2				−0.298	
χ^2	0.004	0.001	0.003	0.004	0.002

[a] Equation 2: $Y = [(A1 - A2)/[1 + (X/Xo)^P] + A2$; equation 3: $Y = (A1 - A2)/(1 + e^{(X-Xo)/dx}) + A2$; equation 4: $Y = A/(1 + e^{-K(X-Xc)})$; equation 5: $Y = 0{,}6843 - (A2 - A1)F/(1 + 10^{(X - \log X1)}) - [(A2 - A1)(1 - F)/(1 + 10^{(X - \log X2)})]$.

Imperatorin displayed a two-part dose–response curve. At lower concentrations the activity trend followed a pattern comparable to that of other FCs, reaching a plateau between 2 and 3 μg/disk. This was followed by a second, much higher plateau above 4 μg/disk, suggesting that more than one FC discrimination system assists the insect in its feeding choice. This abnormal behavior required a special treatment of imperatorin in its evaluation as synergist (see below). A complex equation (equation 5, Table 3) was necessary to accommodate all the data points. However, by using only the data below 3 μg/disk, it was possible to fit the recorded activity to a much simpler and accurate mathematical representation (equation 6; Figure 2D). Thus, imperatorin at concentrations below 3 μg/disk was used.

$$[1 - \mathrm{FR}_{50}] = 0.060 + 0.190X - 0.041X^2 \tag{6}$$

$r^2 = 0.9919$; $P < 0.0008$.

The dose–response curve of bergapten (Figure 2E) showed a similar inflection at 1.5 μg/disk that demanded a double mathematical treatment: a Gompertz sigmoid for those points in the $0 < X < 1.5$ μg/disk range (equation 7) and a logistic sigmoid fitting for points above this value (equation 2, Table 3).

$$Y = Ae^{-e^{-[K(X - X_c)]}} \tag{7}$$

where $A = 0.610$, $K = 12.169$, $X_c = 0.365$, and $\chi^2 = 0.001$.

The phagodepressant activity of angelicin has been examined in only a few polyphagous insects (Muckensturn et al., 1981) and has not previously been examined in *S. littoralis*. Its dose–response data followed a sigmoidal Boltzman distribution, in parallel with xanthotoxin, but at a much reduced level (equation 3, Table 3; Figure 2F).

Activity of Binary Mixtures. The expected feeding deterrence activity of binary mixtures of FCs was calculated by the algebraic sum of individual activities represented by their particular best-fit equations. The calculated composition of the mixtures was set at 10% intervals between 0 and 100% of one of the components, keeping their added concentration *K* constant. From the estimated result, the concentration range to be used experimentally was determined such that the total activity was at most 90% of the maximum activity in terms of $1 - FR_{50}$ (Alonso-Amelot and Calcagno, 2000). This procedure allowed us to detect any significant synergistic effect without reaching the 100% response level where the insect's sensory system would presumably become saturated. Estimated dosages for each FC combination were 2 μg/cm^2 for imperatorin–xanthotoxin, 3 μg/cm^2 for imperatorin–psoralen and angelicin–xanthotoxin, and 5 μg/cm^2 for angelicin–psoralen. Three dosages (*K*) were used for the imperatorin/bergapten combination (1, 2, and 3 μg/cm^2) to evaluate if the purported synergism was dose-dependent and to assess the effect on the total insect feeding behavior of the dose–response curve discontinuity of bergapten.

In the case of imperatorin–xanthotoxin, moderate synergism became evident only in the 40–75% imperatorin range (Figure 3A), but only the point at 40%, whose coactivity coefficient (CA) was 121, was statistically distinguishable from the additive value. Imperatorin–psoralen mixtures were studied at greater *K* (3 μg/cm^2) because of the lower activity of psoralen and the limitations imposed by the dose–response curve distortion above this value for imperatorin (Figure 2C). A correlation analogous to that of imperatorin–xanthotoxin was obtained (Figure 3B) with a similar zone of synergism. The maximum effect was found at 75% imperatorin (CA = 153).

In the smoothly distributed experimental points of the imperatorin–bergapten mixtures, two zones became apparent (Figure 4A). At $K = 1$ μg/cm^2, synergism occurs at >60% imperatorin, whereas lower imperatorin proportions lead to antagonism. The synergistic range is narrower (>80% imperatorin) at $K = 2$ μg/cm^2 and the recorded $1 - FR_{50}$ values in the plateau zone become undifferentiated from the addition curve. A similar behavior in the synergistic range was recorded for $K = 3$ μg/cm^2, but activities in the plateau were significantly antagonistic. In all three cases, the bergapten concentration where synergism was observed was only around 0.25 μg/cm^2, just below the activity threshold of this compound (Figure 2E). Thus, imperatorin must be a synergist for bergapten in this dose range.

Our data indicate that mixtures of linear FCs, xanthotoxin, psoralen, and bergapten with imperatorin at 40% or greater of the latter may exert a greater

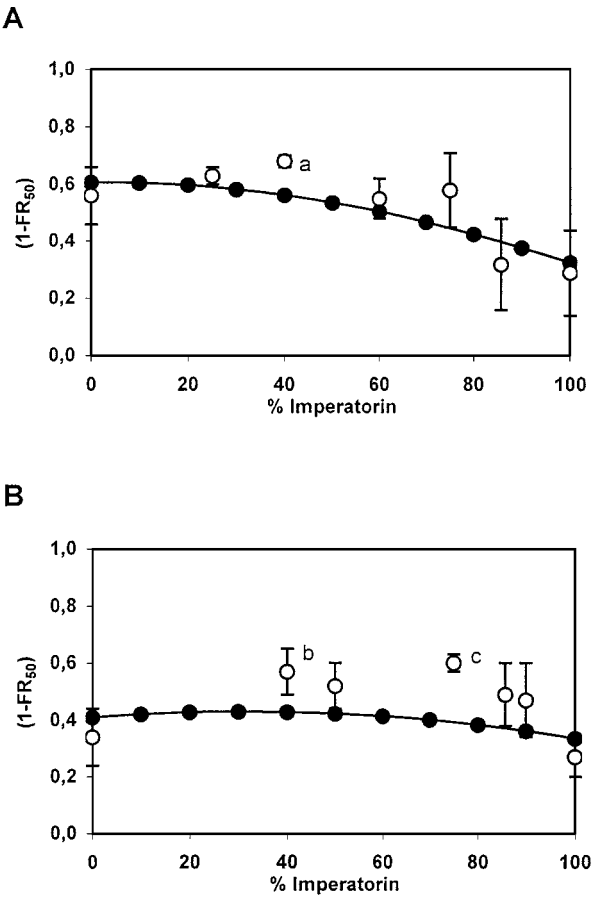


FIG. 3. Effect of binary mixtures of linear FCs in feeding deterrence activity on *S. littoralis*. Experimental (○) and calculated ((●) solid line) additive activities of mixtures of (**A**): imperatorin + xanthotoxin at $K = 2\mu g/cm^2$ and (**B**): imperatorin + psoralen at 3 $\mu g/cm^2$ showing moderate synergism in the 40–75% imperatorin range. Significant data points in lower captions ($\alpha = 0.05$ Tukey). (**A**) a: CA = 121; (**B**) b: CA = 131; c: CA = 153. Bars represent SEM.

antifeedant effect on a generalist herbivore such as *S. littoralis* than the individual compounds. This conclusion is in agreement with recent work on FC effects on *S. exigua*, although imperatorin was not examined (Berdegué et al., 1997). Conversely, xanthotoxin alone possessed a higher feeding deterrency activity against *Helicoverpa zea* than the FC mixture naturally found in *Pastinaca sativa* (Berenbaum et al., 1991), indicating a moderate antagonism. This also fits the

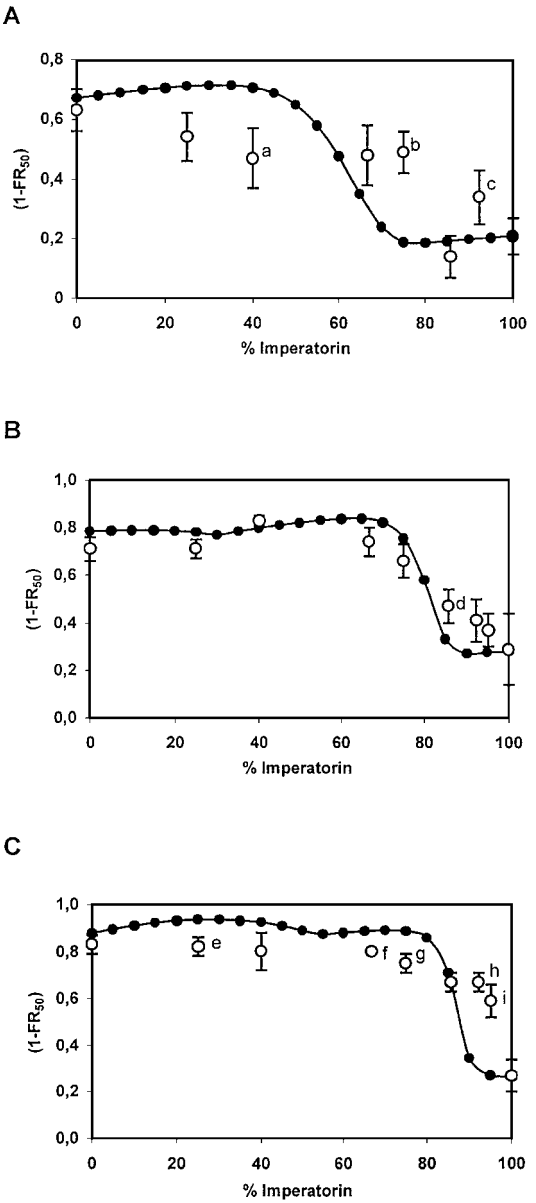


FIG. 4. Effect of binary mixtures of imperatorin and bergapten in feeding deterrence activity on *S. littoralis* at different total doses. Experimental (○) and calculated (●) additive activities of mixtures showing significant antagonistic and synergistic zones. Significant data points in lower captions ($\alpha = 0.05$, Tukey). (**A**) $K = 1\ \mu g/cm^2$; a: CA = 67; b: CA = 257; c: CA = 169. (**B**) $K = 2\ \mu g/cm^2$, d: CA = 151. (**C**) $K = 3\ \mu g/cm^2$, e: CA = 88; f: CA = 90; g: CA = 85; h: CA = 240; i: CA = 217. Bars represent SEM.

portion of our dose–response regressions where antagonism occurs, such as in the imperatorin–bergapten combinations. This might well be the case with xanthotoxin in complex combinations such as those reported by Berenbaum et al. (1991) in *P. sativa*.

Thus, it might be advantageous for the parent plant to synthesize and accumulate various FCs, including imperatorin, to fend off generalist herbivorous insects. Because not only the presence but also the concentration and, more importantly, the proportions of these compounds appear to be essential in the synergistic effect, it will be necessary to determine quantitatively the relative amounts of these materials in a wide range of natural systems that find protection by use of FCs. Data at present are probably insufficient to draw conclusions in this regard, but some evidence is clearly suggestive. Studies on *Psoralea* showed the presence of linear and angular FCs at relatively constant ratios in various plant parts (e.g., Cappelletti et al., 1992). Their accumulation in whole fruits is reported at a 0.3–0.4 psoralen–angelicin ratio in *P. cinerea*, *P. onobrychys*, and *P. macrostachya*. This combination has been interpreted as a chemical defense of the reproductive organ. In the latter two species, although the total concentration of FCs varies with seed maturation, the psoralen–angelicin ratio changes only marginally (Innocenti et al., 1984; Cappelletti et al., 1992). This pattern, however, may not be universal within this genus, as *P. lacnostachys*, and *P. pustulata* contain these FCs in a 2.0–2.5 ratio (Innocenti et al., 1984).

This compound configuration in the natural systems led us to examine the possible synergistic effect that angelicin, the least active among the compounds examined, has in combination with psoralen. Mixtures yielded a clear synergistic effect in the 20–80% angelicin range (Figure 5A). At $K = 5\ \mu g/cm^2$, a 0.3–0.4 psoralen–angelicin ratio furnished a CA = 124–125. Similarly, a 2.0–2.5 psoralen–angelicin ratio produced a CA = 132–133, both corresponding to a detectable increase in feeding deterrence capacity. Therefore, there may be considerable latitude in the proportion of these compounds that may yield increased protection against herbivory by generalist insect herbivores.

There are some plants that also contain xanthotoxin and angelicin such as *Pastinaca sativa* (Berenbaum et al., 1991; Berenbaum and Zangerl, 1993). It is conceivable that synergism between these compounds could occur. However, using $K = 3\ \mu g/cm^2$ and varying the xanthotoxin–angelicin ratio, our tests indicated that feeding deterrence activity against *S. littoralis* was similar to the calculated additive curve (Figure 5B), so no synergism was in effect. This result is consistent with the behavior of *Papilio polyxenes* facing the same combination of xenobiotic chemicals in *P. sativa* (Berenbaum and Zangerl, 1993). The FC mixture was more toxic than the individual compounds, but feeding deterrence capacity was not greater. It is clear that the synthesis of selected FCs in one organism may be derived from the added protection that these compounds confer to the host plants as mixtures. Other

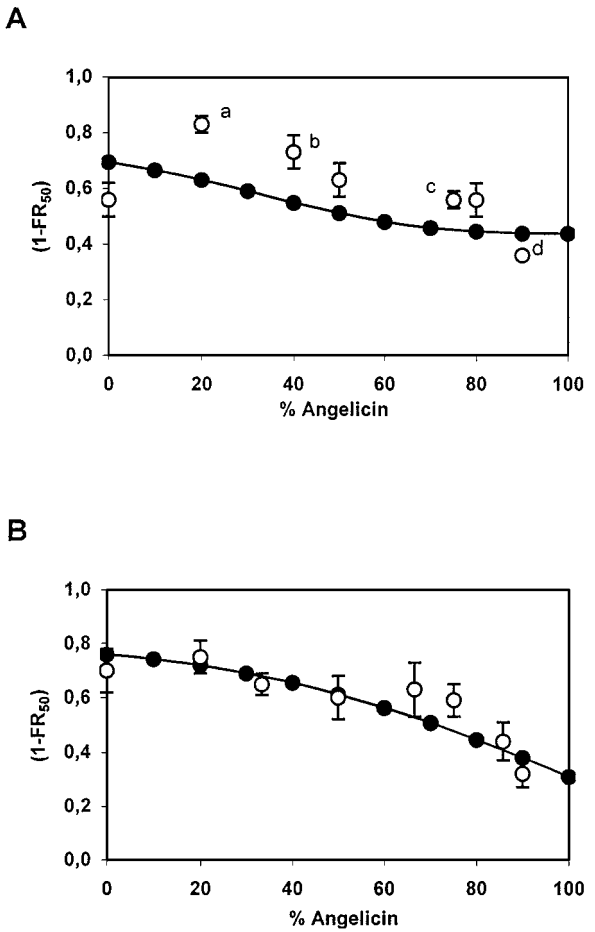


FIG. 5. Effect of binary mixtures of angular and linear FCs in feeding deterrence activity on *S. littoralis*. Experimental (○) and calculated ((●) solid line) additive activities of mixtures of (**A**) angelicin and psoralen at 5 μg/cm^2, showing clear synergism in the 20–80% angelicin range, significant data points in lower captions ($\alpha = 0.05$, Tukey); a: CA = 132; b: CA = 133; c: CA = 124; and (**B**) angelicin and xanthotoxin at $K = 3$ μg/cm^2 (showing no significant synergism). Bars represent SEM.

FC combinations, deemed nonsynergistic at present, may prove to be so in other insects.

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OVIPOSITION RESPONSES BY HESSIAN FLY, *Mayetiola destructor*, TO WHEATS VARYING IN SURFACES WAXES

DANIEL E. CERVANTES, SANFORD D. EIGENBRODE,* H-J. DING, and NILSA A. BOSQUE-PÉREZ

Department of Plant, Soil, and Entomological Sciences
University of Idaho
Moscow, Idaho 83844-2339

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Abstract—Leaf waxes are known to contain oviposition stimulants for Hessian fly. In dual-choice tests comparing seedlings of each of three nonglaucous lines of wheat with its corresponding glaucous line, Hessian flies laid similar numbers of eggs on each genotype. However, when plants from these genotypes were tested at the flag-leaf stage, Hessian flies deposited 25–100% more eggs on the nonglaucous genotype compared to normal wax genotype in each pair. Leaf waxes extracted from the genotypes in one of these pairs (Avalon and nonglaucous Avalon) at the seedling and the flag-leaf stage and tested on paper leaf models elicited oviposition responses similar to those observed on the intact plants. Scanning electron microscopy showed differences in crystal density on nonglaucous and glaucous genotypes only at the flag-leaf stage. Gas chromatography–mass spectrometry of the leaf wax extracts showed that fly oviposition responses were associated with differences in the chemical composition of the waxes. This is the first report of Hessian fly oviposition responses varying among genotypes of wheat with different surface wax composition.

Key Words—Cecidomyiidae, host plant resistance, antixenosis, oviposition preference, plant surface wax, *Triticum aestivum*.

INTRODUCTION

The Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae), is a major pest of wheat (*Triticum aestivum* L.) throughout many wheat growing areas of the world (Barnes, 1956; Ratcliffe and Hatchett, 1997). Hessian fly also infests several other cereal crops, including barley (*Hordeum vulgare* L.), rye (*Secale*

*To whom correspondence should be addressed. E-mail: sanforde@uidaho.edu

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cereale L.), and triticale, and several noncereal grasses (Harris et al., 1996). This insect continues to be problematic for wheat production in most of North America, and during the last two decades has become increasingly important in the Pacific Northwest (Pike et al., 1993; Ratcliffe et al., 2000).

Resistant cultivars are the most effective control method for Hessian fly (Ratcliffe and Hatchett, 1997). Deployed resistance to the fly and all known sources of resistance depend upon antibiosis conditioned by single genes (Ratcliffe and Hatchett, 1997). The high level of mortality imposed on first-instar larvae by resistant varieties exerts a strong selection pressure on Hessian fly populations, resulting in the evolution of Hessian fly biotypes unaffected by resistant genes and posing a risk to the durability of resistant varieties (Ratcliffe and Hatchett, 1997; Ratcliffe et al., 2000). Resistance other than antibiosis could provide a means for increasing the durability of resistance to Hessian fly (Gould, 1986).

Harris and colleagues (Harris and Rose, 1989, 1990; Foster and Harris, 1992; Harris et al., 1993; Kanno and Harris, 2000a,b; Morris et al., 2000) have conducted extensive investigations of the oviposition behavior of Hessian fly. Their research has shown that host finding and oviposition by Hessian fly is influenced by visual, tactile, and chemical cues from the plant, especially the composition of plant surface waxes. Foster and Harris (1992) showed that Hessian flies lay significantly more eggs on paper strips impregnated with epicuticular wax extracts of wheat than on wax-free controls, or strips impregnated with epicuticular waxes from the nonhost, oats. Recently, Morris et al. (2000) reported that *n*-octacosan-1-aldehyde (1-octacosanal) and 6-methoxy-2-benzoxazolinone (MBOA) in wheat seedling surface waxes were major oviposition stimulants for a New Zealand population of Hessian fly.

If wax composition influences Hessian fly oviposition preference, it might be possible to alter wheat wax composition genetically to provide the crop with some protection against the fly. There are no reports of Hessian fly responses to wheat genotypes varying in surface waxes. Working on a group of winter wheat genotypes, Lowe et al. (1985) identified genotypes that lacked a waxy bloom. These were named nonglaucous (NG) Avalon, NG Brigand, and NG Virtue after their respective normal-wax or glaucous parental lines Avalon, Brigand, and Virtue. The NG lines were found to be resistant to the English grain aphid, *Sitobion avenae* F. (Aphididae) by Lowe et al. (1985). Mutations that reduce waxy bloom in wheat and other crops typically reduce the amount of wax (wax load) and the density of wax crystals on a leaf area basis, and alter wax chemical composition (Macey and Barber, 1970; Holloway et al., 1977; Avato et al., 1984; Eigenbrode et al., 1991; Eigenbrode and Pillai, 1998).

We obtained the three NG wheat genotypes and their glaucous counterparts used in the Lowe et al. (1985) study to examine their effects on oviposition by the Hessian fly. We hypothesized that oviposition would be altered on NG plants, either because of the lower loads of stimulatory waxes or because the waxes on NG

plants have altered amounts of oviposition stimulants and/or deterrents for the fly compared to normal wax plants. We examined this hypothesis using free-choice bioassays with plants at the seedling stage and the flag-leaf stage. We tested flag-leaf stage plants because the NG state in the lines we used is only detectable at this stage. We tested plants at the seedling stage because this is most vulnerable to the fly and because the effects of the wax mutations on seedling stage wax composition were unknown. We included a known nonhost, oats, as a negative control in the bioassays with intact seedlings. Based on results of the bioassays with intact plants, we extracted leaf waxes from one pair of wheat genotypes, applied them to paper leaf models, and conducted free-choice bioassays to examine the effect of wax chemistry on Hessian fly oviposition. We characterized the waxes used in this bioassay chemically using gas chromatography/mass spectrometry. Because fly oviposition also might be affected by the differences in crystalline morphology of the waxes on normal and NG types, we examined the surfaces of the plants with scanning electron microscopy (SEM).

METHODS AND MATERIALS

Insects. Hessian flies were obtained from a laboratory colony established with individuals collected from wheat in the summer of 1998 in Lewiston, Idaho. Biotypes GP, E, F, and G make up 83% of the Hessian fly field populations in Lewiston (Ratcliffe et al., 2000). The laboratory colony was determined to have similar frequencies of each biotype (R. Ratcliffe, personal communication). Hessian fly biotypes are characterized by their virulence against a number of resistance genes (Ratcliffe and Hatchett, 1997) and are not known to differ in their oviposition preference. The colony was maintained in the laboratory on the susceptible cultivar Centennial at $21 \pm 4^{\circ}$C, and 16L : 8D, using a method modified from Foster et al. (1988). When plants reached the two-leaf stage, they were infested with 50 mated female Hessian flies per cage over a five-day period. Mated females are distinguished from virgin ones by their characteristic calling posture (Bergh et al., 1990). After oviposition, eggs hatched and larvae developed on wheat plants. Adults emerged after ca. 28–30 days.

Wheat Genotypes. Three sets of winter wheat genotypes (Avalon, Brigand, Virtue, and NG variants from each genotype) and oats (cv. Coast Black) were tested for oviposition preference by Hessian fly. At maturity, the NG variants of each wheat genotype have a distinctly reduced waxy bloom. Seed of Avalon, Virtue, Brigand, and Coast Black oats was obtained from the USDA National Small Grains Collection, Aberdeen, Idaho. Seed of NG Avalon, NG Virtue, and NG Brigand was obtained from the John Innes Center, Norwich, England. Seed of all entries was increased in the greenhouse to obtain sufficient material for testing. For experiments, plants were grown in the greenhouse ($20 \pm 2^{\circ}$C, 16L : 8D) using

supplemental lighting provided by Son Agro 430-W high-pressure sodium lamps (Phillips, Somerset, New Jersey). Plants to be used at the flag-leaf stage were subjected to a six-week vernalization period (4°C, 8L : 16D).

Seedling Stage Bioassays. Free choice tests were conducted in the greenhouse (20 ± 2°C, 16L : 8D). In three separate experiments, comparisons were made between each glaucous wheat genotype and its NG variant. Experiments were arranged as randomized complete block designs with 10 replications (1 cage = replication). Plexiglas cages (51 × 50 × 51 cm) were used for tests. The bottom part of the cage was filled with soil mixture [6 : 1 ratio of Sunshine mix #1 (Sun Gro Horticulture, Bellevue, Washington) and sand], and seeds were planted directly in the soil. For the tests with Avalon and Virtue and their NG variants, each cage contained one row of each genotype (seven plants per row, rows spaced 10 cm, plants spaced 3.5 cm). Genotypes were assigned to rows randomly. When plants reached the three-leaf stage (Zadoks scale 12) (Zadoks et al., 1974), a single mated female obtained from the laboratory colony was released into each cage (09:00 hr) and allowed to oviposit for 6 hr. At the end of the oviposition period, all plants were cut at the base, and Hessian fly eggs were counted using a dissecting microscope. For the test comparing Brigand, Brigand NG, and oats, five mated females rather than one were released per cage, and each row contained eight plants per genotype rather than seven.

Flag-Leaf Stage Bioassays. The distinctly reduced waxy bloom on NG variants was only discernible on plants after vernalization and nearing emergence of the flag leaf. Although under field conditions most oviposition by Hessian fly occurs on seedlings, Painter (1951) reported that females lay eggs on the flag leaf of winter wheat plants, if available, during the oviposition period. For these reasons, we also conducted oviposition tests using flag-leaf stage wheat plants (Zadoks scale 50) (Zadoks et al., 1974). Each glaucous genotype and its NG variant was tested in a separate experiment in the greenhouse and under the same environmental conditions as for the seedling stage experiments. Plexiglas cages (92 × 50 × 51 cm) contained two potted plants (15-cm pots) each of the glaucous genotype and its NG variant. Plants were located randomly within a cage and each experiment had 10 replications. When plants reached the flag-leaf stage, eight mated females were released into each cage and allowed to oviposit for 24 hr, after which eggs were counted using a dissecting microscope. Eggs were counted separately on flag leaves and the entire plant.

Leaf Model Bioassays. Bioassays using leaf models impregnated with leaf wax extracts, as described by Harris and Rose (1990), were conducted to determine the role of epicuticular waxes in fly preference. We tested the activity of waxes extracted from Avalon and NG Avalon because Hessian fly discrimination between these two was stronger and more consistent than between the other two pairs of glaucous and NG genotypes. Leaf models consisted of 10- × 0.5-cm strips of yellow copy paper (Canary Yellow, Color Source, Boise Cascade, Boise, Idaho).

Waxes extracted from Avalon or NG Avalon (see extraction methods below) were dissolved in hexane and applied to the models at physiological concentration (PC) on a mass per leaf area basis. Physiological concentrations were determined by weighing extracted waxes from known leaf area samples of Avalon and NG Avalon plants. Leaf models treated with hexane served as controls. Acetate cages (14 cm diam. $\times$ 15.5 cm high) with a screen top were used for bioassays. The bottom part of the cage was covered with fine sand to a depth of ca. 8 mm and the inner sides were lined with blue paper (Morris et al., 2000). Just before the flies were introduced, the sand was moistened with 30 ml of water. For tests using flag-leaf wax extracts, five leaf model treatments were positioned randomly in each cage: models treated with wax extracts of Avalon glaucous or Avalon NG wax at Avalon PC (23 μg/cm^2), models treated with wax extracts of Avalon glaucous or Avalon NG wax at NG Avalon PC (13 μg/cm^2), and a hexane-treated model as a control. Ten female and two male Hessian flies were released in each cage (09:00 hr) and eggs were counted 6 hr later using a dissecting microscope. Tests using seedling wax were conducted similarly, but only three leaf model treatments were used in each cage: models treated with Avalon and NG Avalon seedling waxes at the same concentration (1.6 μg/cm^2) and a hexane-treated model as a control. This concentration was the average PC of waxes on Avalon and NG Avalon at the seedling stage, which are similar. Five female flies and two males were released per cage, and eggs were counted as described above. Cages were kept in a greenhouse at 20 $\pm$ 2°C. Each test was replicated 12 times.

Scanning Electron Microscopy. To characterize the morphology of the leaf surface waxes, scanning electron micrographs (SEM) of upper leaf surfaces of each genotype were taken at the Electron Microscopy Center, Washington State University, Pullman, Washington. In our experiments with flag-leaf-stage plants, and as previously reported for seedlings (Harris and Rose, 1989; Kanno and Harris, 2000a), the upper leaf surfaces of plants receive virtually all of the Hessian fly eggs. Samples were prepared for imaging as in Eigenbrode et al. (1998). Tissue samples of each genotype were taken from seedling and flag-leaf-stage wheat plants grown in the greenhouse. Samples (4 $\times$ 4 mm) were cut and placed on microscopy stubs and transferred into a drying chamber at -20°C. After two days, dried samples were sputter-coated with gold for nine, 10-sec intervals (for a total of 90 sec) at 10 mA. Micrographs for each genotype (glaucous and NG) and each developmental stage were taken at 15 kV at 5000$\times$ magnification.

Measuring Wax Crystal Density. SEM images of flag leaves were used to estimate the density of wax crystals on leaf surfaces of the test lines. Four replicate 100-μm^2 areas of the adaxial surfaces were randomly selected and the crystals were counted within them.

Gas Chromatography. Plant waxes were extracted from Avalon and NG Avalon at the seedling and flag-leaf stages with 20-sec hexane washes at room temperature. The relatively short duration of this extraction time was intended

to minimize extraction of internal plant constituents. To avoid extracting internal plant constituents, cut leaf ends were not immersed in the solvent during extraction. Waxes of all leaves of 20 plants within each developmental stage of each genotype were pooled. Each wax extract was analyzed underivatized and derivatized with *N*,*O*-bis(trimethylsilyl) acetamide (Sigma, St. Louis, Missouri), dissolved in hexane, and analyzed separately by gas chromatography–mass spectrometry (GC-MS). The samples were characterized by using a Hewlett-Packard (HP) 6890 GC equipped with a HP5973 quadrupole mass selective detector interfaced with HP Chemstation software. The GC-MS was equipped with an SP-1, fused silica capillary column (30 m × 0.2 mm ID) (Sigma-Aldrich) and operated in splitless mode with injector temperature at 320°C. The oven temperature program was 80°C for 1 min, 15°/min to 260°C, hold 10 min, then 5°C/min to 320°C, and hold 15 min. Identification of the components was based on mass spectra and retention times of authentic standards for some of the components (*n*-pentacosane, *n*-heptacosane, *n*-nonacosane, *n*-triacontane, and trimethyl-silyl esters and ethers of tetradecanoic acid, hexadecanoic acid, octadecanoic acid, hexacosanoic acid, *n*-docosan-1-ol, *n*-octacosan-1-ol, and *n*-triacontan-1-ol). Quantitation was based on the total ion count per peak, adjusted using response factors for standard alkanes, free fatty acids, primary alcohols, and wax esters (based on response of palmitic acid behenyl ester). Quantitation of fatty acids and alcohols was based on their TMS esters and ethers from the derivatized samples; all other components were quantified based on underivatized samples. Total wax load (micrograms per square centimeter of leaf area) was calculated based on the area of the leaves extracted for each sample. Because wax loads differed among the lines, relative composition (percentage of total load) of the wax components was calculated from the raw data. The ratio of percent composition for each component in NG Avalon and Avalon was also calculated for each component (percentage in NG Avalon to percentage in Avalon). Seedling waxes and flag-leaf waxes were analyzed in the same manner.

Morris et al. (2000) identified 1-octacosanal and MBOA as oviposition stimulants for the Hessian fly. To ensure that our methods were efficient for these known oviposition stimulants, we conducted pilot tests using our hexane extraction method (20 sec in hexane) and extraction with CH_2Cl_2 at 10°C for 60 secs (Morris et al., 2000). As another check on our methods, we included Otane seedlings (seeds courtesy of M. Harris, North Dakota State University, Fargo, ND). Otane is a widely grown wheat variety in which 1-octacosanal and MBOA were identified as oviposition stimulants for Hessian fly (Morris et al., 2000). These extracts were analyzed by GC-MS and compared.

Statistical Analysis. The numbers of eggs per female per plant for the plant bioassays and the numbers of eggs per strip for leaf model assays were compared with analysis of variance (ANOVA) using the GLM procedure in SAS (SAS Institute, Cary, North Carolina). For the Avalon seedling leaf model tests, two contrasts were conducted: (1) NG Avalon vs. Avalon and (2) wheat wax extracts vs. hexane

control. For the Avalon flag-leaf-stage leaf model tests, three contrasts were conducted: (1) NG Avalon vs. Avalon, including both high and low PCs; (2) high PC (23 μg/cm^2) vs. low PC (13 μg/cm^2), and (3) all wheat wax extracts vs. hexane control.

RESULTS

In tests with seedlings, Hessian fly females laid eggs only on wheat plants; no eggs were laid on oats. The mean number of eggs laid per female per plant was not different between NG wheat lines and the glaucous lines: Avalon lines ($F = 1.24$, $P = 0.31$), Virtue lines ($F = 1.40$, $P = 0.28$), and Brigand lines ($F = 1.41$, $P = 0.24$) (Figure 1).

In tests with flag-leaf-stage plants, Hessian fly laid more eggs per female per plant ($F = 20.31$, $P < 0.001$) and per flag leaf ($F = 10.84$, $P = 0.0026$) on NG Avalon than on Avalon, and more eggs per female per plant on NG Virtue than Virtue ($F = 7.64$, $P = 0.009$) (Figure 2). The number of eggs laid on the flag leaves of NG Virtue and Virtue did not differ ($F = 0.03$, $P = 0.859$) (Figure 2). The number of eggs laid per female per plant on NG Brigand and Brigand plants did not differ ($F = 1.29$, $P = 0.26$), but more eggs per female were laid on NG Brigand flag leaves than on Brigand flag leaves ($F = 15.77$, $P = 0.001$) (Figure 2).

The oviposition patterns by Hessian fly on paper leaf models treated with waxes from Avalon and NG Avalon were similar to those that occurred on the Avalon and NG Avalon intact plants. There were no significant differences in the number of eggs laid on leaf models treated with seedling wax extracts of NG Avalon and Avalon (Figure 3). Models treated with only hexane received fewer eggs than models treated with wax extracts ($F = 7.24$, $P = 0.002$). Models treated with NG Avalon flag-leaf wax extracts received twice as many eggs as models treated with waxes from Avalon flag-leaf wax extracts (Figure 4) ($F = 22.54$, $P < 0.001$). There was no effect of wax physiological concentration on the number of eggs laid within or across the wax extract ($F = 0.20$, $P = 0.6604$). Together, flag-leaf wax extract-treated models received more eggs than the hexane-only control ($F = 16.19$, $P < 0.001$).

Scanning electron micrographs of adaxial leaf surfaces of seedlings showed no visible differences in wax (Figure 5). Crystal densities on NG Avalon (273 ± 13) were similar to those on Avalon (286 ± 10). Crystal densities on NG Brigand (172 ± 34) were lower than those on Brigand (294 ± 25) (t test $P = 0.03$). Crystal densities on NG Virtue (299 ± 28) appeared to be higher than on Virtue (230 ± 28), but the difference was not significant ($P = 0.14$).

Waxes extracted from NG Avalon and Avalon apparently differed in chemical composition, but the differences appeared more pronounced between the flag-leaf waxes than between the seedling waxes (Table 1). Our data are from pooled samples

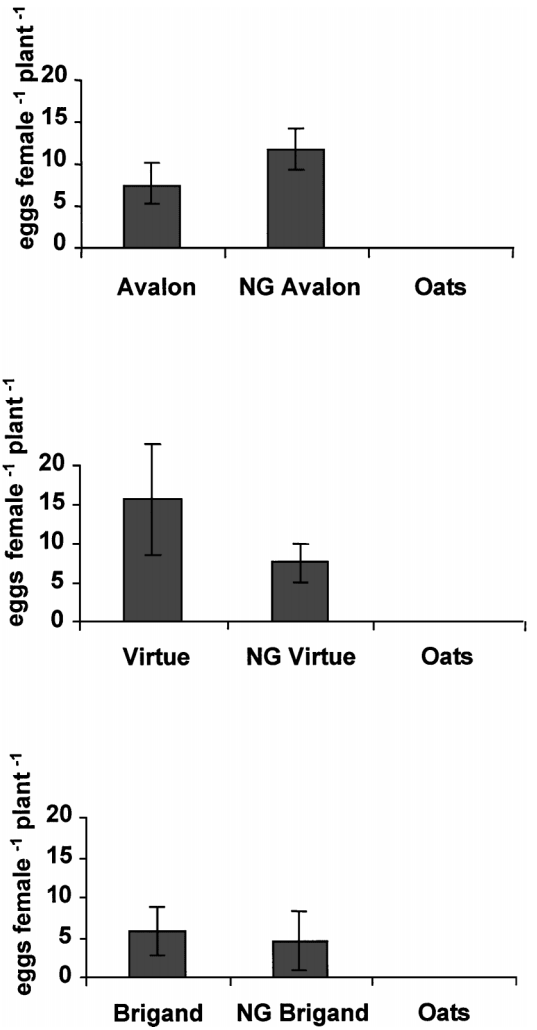


FIG. 1. Mean number of eggs laid per female Hessian fly per plant in choice tests in a greenhouse among seedling stage oats and wheat plants that vary in surface wax composition. NG = nonglaucous. Bars are standard errors of the means.

of 20 plants of each line at each developmental stage, but are not replicated, preventing statistical comparisons. Seedling waxes were dominated by hexadecanoic acid, 1-octacosanol, hentriacontane, and wax esters, but included 26 components, including six unidentified wax esters (each with a C_{28} chain length acid moiety).

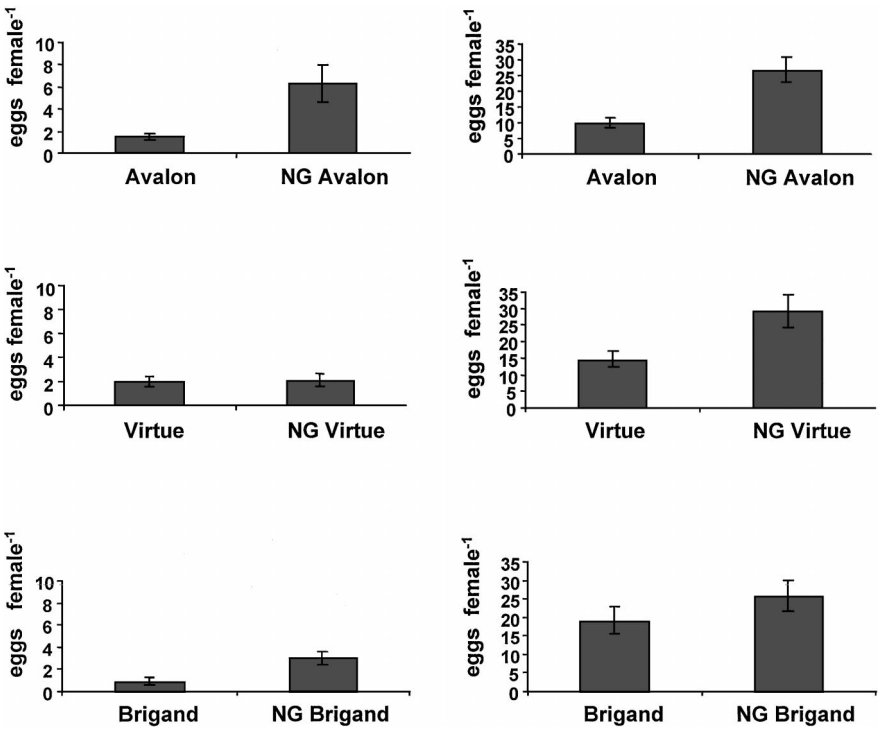


FIG. 2. Mean number of eggs laid per female Hessian fly in choice tests in a greenhouse among flag-leaf-stage wheat plants that vary in surface wax composition. Left panels show data from flag leaves of the plants; right panels show data from entire plants. Bars are standard errors of the means.

The percent compositions of all components were similar in Avalon and NG Avalon seedling waxes. The NG Avalon/Avalon ratio of percent composition for individual components ranged between 0.8 and 2.7. The largest NG Avalon/Avalon ratios were for tricosane and 3-methyl-tricosane, both of which were minor components (<1% of total wax based on GC-MS). 1-Octacosanal composition was similar in the two waxes (NG Avalon/Avalon ratio = 0.96). MBOA was detectable at trace levels in NG Avalon and Avalon seedling waxes.

Compared with seedling waxes, flag-leaf waxes had lower concentrations of free fatty acids (mean of 3.2% as compared with 21.1% on seedling waxes), detectable concentrations of β-diketones, a shift of the dominant hydrocarbon to *n*-nonacosane from *n*-hentriacontane, a nearly fivefold increase in wax esters, and a greater number of detectable components, including five to eight wax esters.

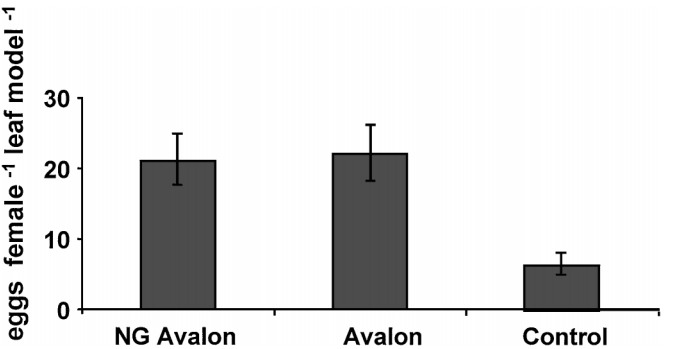


FIG. 3. Mean numbers of eggs deposited per female Hessian fly in choice tests in a greenhouse among paper leaf models treated with surface wax extracts of seedling-stage Avalon, seedling-stage NG Avalon (both at a concentration of 1.6 μg/cm^2), or hexane. Bars are standard errors of the means.

MBOA was not detected in flag-leaf waxes. The NG Avalon/Avalon ratio of percent composition for individual components ranged between 0 and 4.5. Altogether, 13 components were relatively higher and 19 components were relatively lower in NG Avalon flag-leaf waxes as compared with Avalon flag-leaf waxes. The largest ratios (>2) were for 9-octadecenoic acid (oleic acid), *n*-dodecanal, *n*-tetracosanol, and nonadecane-2,4-dione. Notably small NG Avalon/Avalon ratios (<0.05)

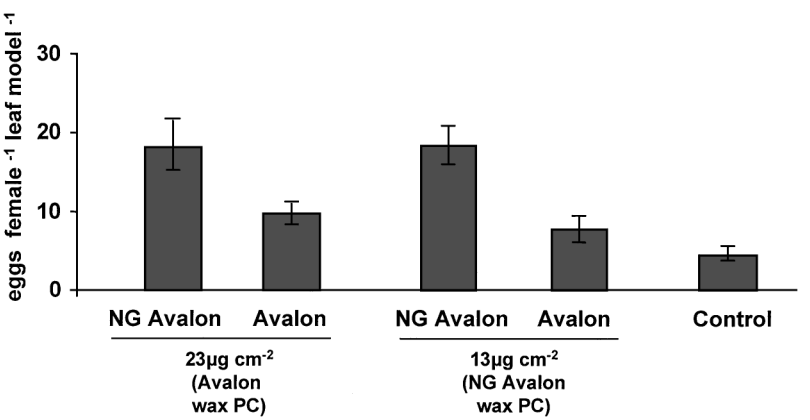


FIG. 4. Mean numbers of eggs deposited per female Hessian fly on paper leaf models treated with surface wax extracts of flag-leaf stage Avalon or flag-leaf stage NG Avalon. Treatments were NG Avalon and Avalon at the Avalon physiological concentration (23 μg/cm^2) per leaf area, NG Avalon and Avalon at the NG Avalon physiological concentration (13 μg/cm^2), and a control treated with hexane. Bars are standard errors of the means.

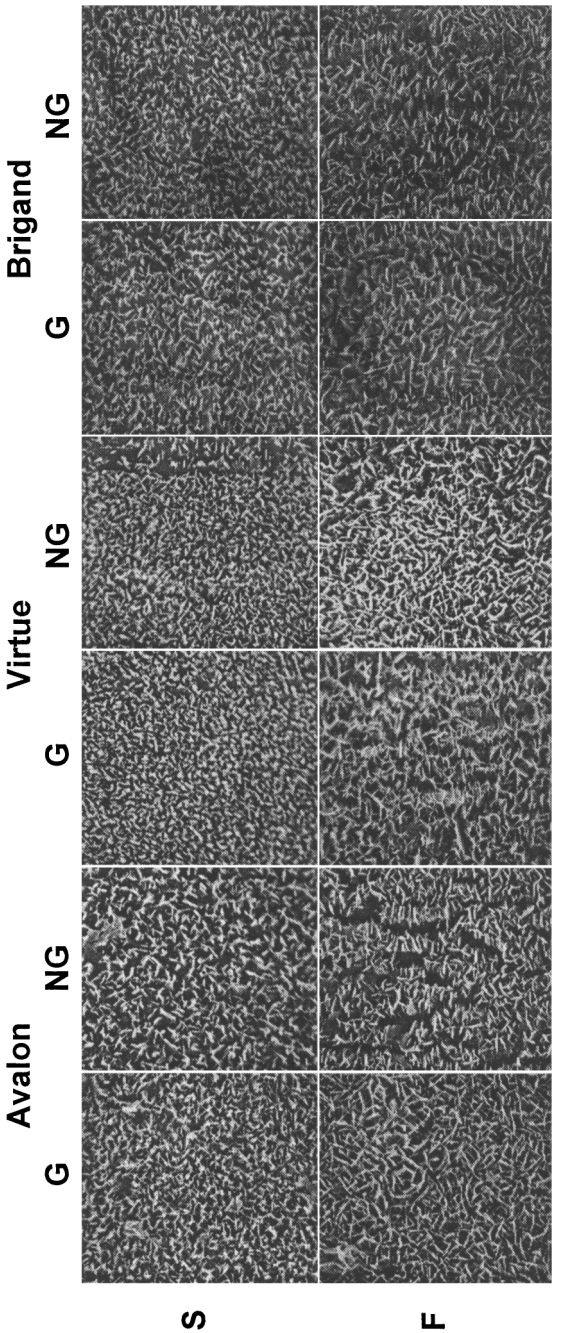


FIG. 5. Scanning electron micrographs of adaxial leaf surfaces of Avalon and NG Avalon (left), Virtue and NG Virtue (center), and Brigand and NG Brigand. G = glaucous, NG = nonglaucous, S = seedling-stage plants, F = flag-leaf-stage plants.

TABLE 1. EPICUTICULAR WAXES FROM SEEDLING AND FLAG LEAVES OF AVALON AND NG AVALON WHEAT

	Flag leaf waxes			Seedling leaf waxes		
	Percent composition		NG Avalon	Percent composition		NG Avalon
Component[a]	Avalon	Avalon	Avalon	Avalon	Avalon	Avalon
n-Alkanoic acids						
Dodecanoic acid	0.03	tr[d]		0.36	0.41	1.15
Octanedinoic acid	tr	tr				
Nonanedinoic acid	0.08	0.08	1.09			
Tetradecanoic acid	0.39	0.19	0.47	1.47	1.54	1.05
Pentadecanoic acid	0.04	0.04	0.95	0.40	0.42	1.04
9-Hexadecenoic acid	0.06	0.05	0.77			
Hexadecanoic acid	1.12	0.39	0.35	16.88	15.00	0.89
Heptadecanoic acid	0.04	tr		0.32	0.40	1.26
9-Octadecenoic acid	0.06	0.27	4.45			
Octadecanoic acid	0.08	0.10	1.16	2.43	2.60	1.07
Docosanoic acid	0.42	0.26	0.62			
Tetracosanoic acid	0.39	0.34	0.86			
Octacosanoic acid	0.50	0.47	0.95			
n-Alkan-1-ols						
n-Docosan-1-ol	0.26	0.24	0.93			
n-Tetracosan-1-ol	2.77	7.62	2.75			
n-Hexacosan-1-ol	2.23	3.38	1.52	0.37	0.60	1.61
n-Octacosano-1-ol	27.49	37.06	1.35	30.8	36.06	1.17
n-Dotriacontan-1-ol	4.08	5.46	1.34			
Monoketones and β-diketones						
2-Pentadecanone	0.10	0.02	0.23			
7-Hexyl 2-oxepanone[b]	0.70		0.00			
2-Heptadecanone	0.98	0.03	0.03			
Nonadecane-2,4-dione	0.11	0.25	2.22			
Hentriacontane 14,16-dione	11.97	4.80	0.40	tr	tr	

Tritriacontane 14,16-dione	7.87		0.00	tr	tr	
n-Alkanals						
1-Dodecanal	0.07	0.22	3.36			
1-Hexadecanal	0.07	0.02	0.31			
1-Tetracosanal	0.04	0.03	0.84			
1-Hexacosanal	0.08	0.07	0.90			
1-Octacosanal	0.59	0.58	0.98	1.49	1.43	0.96
1-Triacontanal	0.61	0.56	0.92	0.44	0.34	0.77
Hydrocarbons						
n-Eicosane	tr	tr				
n-Heneicosane	0.02		0.00			
n-Docosane	tr	tr				
(*Z*)-9-Tricosene	0.08	0.12	1.47			
n-Tricosane	0.02	0.03	1.22	0.03	0.04	1.36
n-Pentacosane	0.03	0.03	0.82	0.19	0.17	0.91
n-Heptacosane	0.45	0.62	1.37	0.19	0.17	0.91
n-Octacosane	tr	tr				
3-Methyl-tricosane	0.25	0.28	1.11	0.25	0.67	2.74
n-Nonacosane	4.19	5.09	1.21	0.47	0.5	1.07
n-Hentriacontane	1.26	1.34	1.07	35.67	31.38	0.89
n-Tritriacontane	0.42	0.21	0.51	1.62	1.34	0.83
6-Methoxy-2-benzoxazolinone (MBOA)			tr	tr		
Wax esters[c] (no. of peaks)	28.99 (8)	29.40 (5)	1.01	5.68 (6)	6.11 (6)	1.08
Total wax load (μg/cm^2)	23.0	14.6	0.63	2.2	1.9	0.89
Total number of components detectable	50	47		25	25	

[a] Components are presented in their order of elution from GC, within each compound class.
[b] Requires confirmation.
[c] Total load for all detected wax esters; acid moiety C_{28} for all wax esters detected; alcohol moiety variable and not always clearly determinable on the basis of mass spectra.
[d] tr = trace: concentration too low for reliable integration; identification based on detectable diagnostic ions and retention times of an authentic standard (MBOA) or of the same components present in other wax mixtures (diketones).

occurred for tritriacontane 14,16-dione, 7-hexyl 2-oxepanone, heneicosane, and 2-heptadecanone. All were minor components (<1% of total wax), except tritriacontane 14,16-dione and *n*-tetracosan-1-ol. The major component 1-octacosanol (>25% of each wax type) was 1.35-fold higher in concentration in NG Avalon than in Avalon. 1-Octacosanal content was slightly higher in Avalon flag-leaf waxes than on NG Avalon.

Seedling wax extracts of Otane were similar in composition to Avalon seedling wax extracts. However, whereas trace amounts of MBOA occurred in seedling waxes of Avalon and NG Avalon, Otane waxes contained 0.05% MBOA, regardless of the extraction method (hexane 20 sec or CH_2Cl_2 extraction at 10°C for 60 sec). Avalon and NG Avalon contained 1.49% and 1.43% 1-octacosanal, respectively (hexane extraction), or 1.43% and 1.70% 1-octacosanal, respectively (CH_2Cl_2 extraction), while Otane contained 2.42% 1-octacosanal (hexane extraction) or 2.87% 1-octacosanal (CH_2Cl_2 extraction).

DISCUSSION

This is the first report of Hessian fly oviposition responses varying among wheat lines known to have different wax composition. Three different NG variants received significantly more eggs than their glaucous wheat counterparts in dual-choice tests with plants at the flag-leaf stage. However, the same pairs of wheat lines tested at the seedling stage, before differences in glaucousness are apparent, received similar numbers of eggs. Oviposition responses to paper models treated with wax extracts of NG Avalon and Avalon mirrored oviposition responses to the intact plants; models treated with seedling wax extracts of the two lines received similar numbers of eggs, whereas models treated with flag-leaf wax extracts of NG Avalon received significantly more eggs than models treated with flag-leaf wax extracts of Avalon. These results confirm our hypothesis that wax variation in wheat influences Hessian fly oviposition and indicate that wax composition contributes to Hessian fly oviposition responses to the intact plants.

The differences in composition of NG Avalon and Avalon flag-leaf waxes as detected by GC-MS (Table 1) may help explain the greater numbers of eggs laid by Hessian flies on intact plants and models treated with flag-leaf waxes from NG Avalon. We did not carry out statistical comparisons of components. However, candidate components affecting oviposition include those apparently differing in relative percentage in Avalon and NG Avalon flag-leaf waxes. Thirteen components are present in higher concentrations in NG Avalon flag-leaf waxes (NG Avalon/Avalon ratio >1.05, Table 1) and are potential stimulants. Nineteen components are present in higher concentrations in Avalon waxes (NG Avalon/Avalon ratio <0.95) and are potential deterrents. Actual activities of these components are unknown. Other components not detected by GC-MS could also determine

Hessian fly oviposition response to the waxes. Fractionations coupled with additional bioassays are required to identify the active components.

Our study was influenced by prior work showing that Hessian fly oviposition is stimulated by extracted surfaces waxes of wheat seedlings (Harris and Rose, 1989, 1990; Foster and Harris, 1992), culminating most recently in identification of 1-octacosanal and MBOA as oviposition stimulants for the fly (Morris et al., 2000). We thought it possible that differences in the concentrations of these known stimulants would help explain fly responses to NG and glaucous Avalon wheats. 1-Octacosanal and MBOA were present in seedling waxes of Avalon wheats (Table 1), but at concentrations lower than those that occur in Otane wheat from which they were identified as stimulants (Morris et al., 2000; B. Morris, personal communication). Avalon and NG Avalon seedling waxes contained only a trace of MBOA, as compared with 0.05% in Otane seedling waxes. NG Avalon and Avalon seedling waxes contain similar amounts of the oviposition stimulant 1-octacosanal (Table 1), which may be consistent with the similar rates of oviposition on leaf models impregnated with these waxes. MBOA was not detected in Avalon or NG Avalon flag-leaf wax extracts, and 1-octacosanal was present in apparently higher concentrations in the less stimulating extracts of Avalon flag-leaf waxes than in the more stimulating extracts of NG Avalon flag-leaf waxes (Table 1). Therefore, our data indicate MBOA and 1-octacosanal apparently do not explain the different responses by an Idaho population of Hessian fly to NG Avalon and Avalon.

Morris et al. (2000) tested the behavioral activity of long-chain aldehydes other than 1-octacosanal (chain lengths between C_{22} and C_{29}) and reported that all stimulated oviposition by the Hessian fly more than a solvent control and that this activity was greatest for a chain length of 26 carbons. However, Avalon waxes apparently contain a slightly higher percentage of the C_{26} aldehyde than NG Avalon waxes (NG Avalon/Avalon ratio = 0.9, Table 1). The only *n*-alkanal in higher concentrations in NG Avalon waxes is *n*-dodecanal (C_{12}) (NG Avalon/Avalon ratio = 3.36). Morris et al. (2000) did not test *n*-dodecanal. Even if all aldehydes are presumed active, the total concentration of this class does not apparently differ between NG Avalon and Avalon flag-leaf waxes (1.48% on NG Avalon vs. 1.43% on Avalon). Moreover, if the small difference detected is real, it converts to 0.015 $\mu g/cm^2$ on our leaf model bioassay, well below the threshold (0.6 $\mu g/cm^2$) for oviposition response by the Hessian fly to pure 1-octacosanal bioassayed on paper models [threshold calculated from data presented in Morris et al. (2000)]. Thus, differences in *n*-alkanals do not appear to be sufficient to account for the observed greater oviposition on models treated with NG Avalon flag-leaf waxes as compared with Avalon flag-leaf waxes.

It is probable that the Hessian fly responds to mixtures of wax components during host selection. The host range of the flies includes several grasses other than wheat (Harris et al., 1996). Neither the candidate stimulants elevated in NG Avalon nor 1-octacosanal are unique to grasses (Walton, 1990). MBOA and other

benzoxazolinone derivatives also probably occur in waxes of various grasses; one benzoxazolinone derivative has been reported in waxes of corn (Hedin et al., 1993). Indeed, the Hessian fly oviposits on several other grasses, and some triticales stimulate more oviposition than Otane (Harris et al., 1996). Thus, host recognition by the fly cannot be based on presence or absence of these wax components alone. Rather, a combination of components must contribute, as is suggested by the report of Foster and Harris (1992) that combinations of fractions of wheat waxes applied to paper models synergistically stimulate Hessian fly oviposition.

The inconsistencies between our results and those of Morris et al. (2000) do not challenge the validity of their results. First, our conclusions are based on associations between behavior and wax composition, whereas Morris et al. (2000) used bioassay-guided fractionation to identify stimulants for the fly. Second, the concentrations of aldehydes and MBOA in Avalon waxes are lower than Otane waxes, possibly below threshold concentrations for oviposition activity. Third, Morris et al. (2000) used Hessian flies from New Zealand, whereas we used flies from Idaho, and the two populations may differ in their responses to wax constituents. Our results do show that the flies we tested are stimulated to oviposit by wheat wax components other than MBOA and 1-octacosanal.

Morphological attributes of the leaf surface are important stimulants for Hessian fly oviposition (Kanno and Harris, 2000b), and leaf wax structure has been shown to influence oviposition or feeding preference of other herbivores (Uematsu and Sakanoshita, 1989; Bodnaryk, 1992). However, we did not find evidence that wax morphology affected Hessian fly oviposition on our test lines. Seedling waxes did not differ in wax crystal structure and received similar numbers of eggs, and differences in crystal structure between glaucous and nonglaucous lines we tested were inconsistent. NG flag-leaf-stage plants received more eggs in bioassays, but did not differ consistently in crystal density. NG Avalon and NG Brigand had similar crystal densities to their glaucous counterparts, and NG Virtue flag-leaf waxes have greater crystal densities than glaucous types. It is possible that other physical attributes of the waxes that we did not measure influence fly oviposition. Nonetheless, because crystal structure is eliminated in the process of applying extracts to paper models, we can conclude that wax composition alone is an important component in host recognition by the fly and is capable of eliciting responses qualitatively similar to responses to intact plants (Figures 2–5).

Our results raise the possibility of manipulating wheat surface waxes genetically to reduce Hessian fly oviposition. However, the NG lines we examined only elicited reduced Hessian fly oviposition at the flag-leaf stage and not at the economically important seedling stage. If the functional wax characteristics can be discovered, suitable variation in seedling waxes could be sought by screening seedling waxes. In addition, wax production is developmentally regulated as evidenced by the distinct wax types of juvenile and mature wheat (Tulloch and Hoffman, 1973; Bianchi and Corbellini, 1977) (Table 1) and other grasses (Atkin and Hamilton,

1982; Blaker and Greyson, 1988). Surface wax variation in plants appears to depend to a considerable extent on regulatory genes as well as biosynthetic genes (Post-Beittenmiller, 1996; Jenks and Ashworth, 1999). Eventual manipulation of the regulatory genes for wax synthesis in wheat could allow wax variation that influences Hessian fly oviposition on flag leaves to be expressed in the seedling stage in order to influence fly oviposition preferences.

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COMPARING THE VARIATION OF NEEDLE AND WOOD TERPENOIDS IN SCOTS PINE PROVENANCES

A.-M. MANNINEN,[1,*] S. TARHANEN,[1] M. VUORINEN,[2] and P. KAINULAINEN[1,3]

[1]*Department of Ecology and Environmental Science*
University of Kuopio
P.O. Box 1627, FIN-70211 Kuopio, Finland

[2]*The Finnish Forest Research Institute*
Suonenjoki Research Station
FIN-77600 Suonenjoki, Finland

[3]*MTT Agrifood Research Finland*
Plant Production Research, Plant Protection
FIN-31600 Jokioinen, Finland

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Abstract—We determined variation in both the concentration and composition of terpenoids in needles and wood within nine Scots pine (*Pinus sylvestris*) provenances. Seedlings of different provenances representing a 1200-km N–S transect from Estonia to northern Finland were cultivated in Suonenjoki nursery field, central Finland, for seven years. Growth of seedlings and the number of vertical resin ducts in wood were also determined. α-Pinene and 3-carene were the major monoterpenes both in the needles and wood. The total monoterpene concentration was about five times higher in the needles than in the wood. A strong positive correlation was found between proportional quantities of several terpenes of the needles and wood, particularly for 3-carene, sabinene, and terpinolene. The needles contained both labdane-type and tricyclic resin acids, whereas the wood contained only tricyclic ones. The wood had a four times higher tricyclic resin acid concentration than the needles. The highest total monoterpene concentration in the needles and in the wood occurred in the most northern Muonio provenance and in the most southern Saaremaa provenance plants, respectively. The amount of high 3-carene genotype trees decreased among the northern provenances. The wood of the most northern Muonio provenance showed the lowest total resin acid concentration, but provenance did not affect total tricyclic resin acids in the needles. Korpilahti provenance trees from central Finland had the best growth in height. In addition, Korpilahti and Ruokolahti provenance trees showed largest radial growth of stem and smallest number of vertical resin ducts.

*To whom correspondence should be addressed.

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The results suggest that especially the proportional quantity of 3-carene in the needles could be used in estimating the amount of this compound in the wood and vice versa.

Key Words—Terpenes, resin acids, wood, needles, provenance, *Pinus sylvestris*.

INTRODUCTION

Conifers have constitutive and inducible terpenoid-based defense mechanisms that deter insect herbivores and protect against pathogens. The epithelium cells of resin ducts secrete oleoresin, which consists of volatile (mono- and sesquiterpenes) and nonvolatile (resin acids) terpenoids. Terpenoids in needles and wood have an impact on herbivore performance (Leather, 1987; Gershenzon and Croteau, 1991; Manninen, 1999). In Scots pine needles, resin acids are known deterrents of *Schizolachnus pineti* (Homoptera: Lachnidae) (Kainulainen et al., 1996; Manninen et al., 1998b) and *Lygus rugulipennis* (Heteroptera: Miridae) (Manninen et al., 1998a), and also affect the host plant selection of *Neodiprion sertifer* (Hymenoptera: Diprionidae) (Larsson et al., 1986; Saikkonen et al., 1995; Manninen et al., 2000). If the cortical tissue of conifers is wounded, preexisting and induced resin form a physical barrier to seal the wound. Consequently, the concentrations of resin acids in cortical and woody tissues increase near the wound (Gref and Ericsson, 1985; Walter et al., 1989). Oleoresin compounds are an important resistance mechanism against stem-boring bark beetles and fungi associated with them (Bridges, 1987; Paine and Hanlon, 1994; Phillips and Croteau, 1999). Insect attack also induces monoterpene production in wood oleoresin (Valterová et al., 1995) and in phloem (Klepzig et al., 1996).

There is a great variation in the anatomy of oleoresin-producing structures in conifers. These structures range from the most simple isolated resin cells, typical for cedars (*Thuja* sp.), to multicellular resin blisters of true firs (*Abies* sp.), and finally to highly developed resin duct systems of pines (*Pinus*) (Lewinsohn et al., 1991; Phillips and Croteau, 1999). The resin ducts of Scots pine are located in the intercellular space of needle, bark, wood, and root tissues and are surrounded by oleoresin-secreting epithelium cells. In the wood, resin ducts run vertically, mainly among the tracheids of latewood and horizontally in the rays, these being also connected with each other (Wilson and White, 1986). The needles have only lateral resin ducts, and their number and distribution are variable (Esau, 1977).

The composition of oleoresin compounds is genetically controlled (Hiltunen et al., 1975; Gref, 1987; Baradat and Yazdani, 1988), but environmental factors (i.e., light, water, and nutrients) can affect the terpenoid concentrations of conifer needles (e.g., Gleizes et al., 1980; Gref and Tenow, 1987; McCullough and Kulman, 1991; Kainulainen et al., 1996). Terpenoid concentrations may vary among organs of a tree (Gershenzon and Croteau, 1991), between the same organs of individual

trees (Hiltunen et al., 1975; Gref, 1987), and between geographical location and different provenance of trees (Juvonen and Hiltunen, 1972; Muona et al., 1986; Nerg et al., 1994; Tognetti et al., 1997; Manninen et al., 1998b). Furthermore, the relative amount of the different compounds in the oleoresin varies considerably among different tissues of the same tree (Hrutfiord et al., 1974). Studies related to the chemical composition of needle and wood oleoresin within the same pine individuals are rare (Sjödin et al., 1996, 2000; Kleinhentz et al., 1999; Latta et al., 2000). The concentration and composition of oleoresin compounds in the wood has not been intensively studied either (Cown et al., 1992; Coppen et al., 1993).

Earlier, we studied the variation in chemical defense of different Scots pine provenances and whether such variation is related to the performance of insect herbivores damaging needles (Manninen et al., 1998b). The possible connection of wood terpenoids to old house borer (*Hylotrupes bajulus* L.) resistance will also be determined using Scots pine wood blocks originating from the same provenances. It would be a great advantage, if the resistance of Scots pine wood against wood-boring insects could be evaluated by analyzing terpenoid concentration and/or composition in the needles of the same pine individuals. Therefore, the present study aims at: (1) determining the concentration and composition both of needle and wood terpenoids in Scots pine seedlings, (2) finding out whether there is a relation between the concentration and/or composition of terpenoids in needles and wood, (3) determining differences in needle and wood terpenoid content among different provenances growing along a 1200-km north–south transect from Estonia to Finland, and (4) studying the relationships among the concentration of terpenoids, growth parameters, and the number of vertical resin ducts in the wood.

METHODS AND MATERIALS

Growth Conditions. In open pollinated stands of Scots pine (*Pinus sylvestris* L.), seeds were collected from seven geographical locations in Finland and two in Estonia along a 1200-km north–south transect. In the study, a total of nine provenances were used originating from Muonio (MUO) (North latitude 67°56′), Ylitornio (YLI) (66°20′ N), and Suomussalmi (SUO) (65°10′ N) in the north, from Kinnula (KIN) (63°32′ N), Korpilahti (KOR) (62°0′ N), and Ruokolahti (RUO) (61°26′ N) in central Finland, and from Tenhola (TEN) (60°3′ N), Rakvere (RAK) (59°18′ N), and Saaremaa (SAA) (58°22′ N) in the south. The seeds were sown in seedling beds containing peat as a growing medium at the Finnish Forest Research Institute at Suonenjoki Research Station in spring 1991. Plants were fertilized (Puutarhan Y-1 fertilizer, 10:7:14, N:P:K, Kemira) twice a season (120 kg/ha and 80 kg/ha) until spring 1993. In May 1993, 1000 seedlings from each of the nine provenances were planted at Suonenjoki Research Station in the nursery field and randomly divided into five replicate blocks. Every block contained 200 seedlings

representing one provenance. The seedlings were planted in four adjacent lines (50 seedlings in each line and distance between the lines was 0.8 m). The area of each block was 1000 m^2, and the area of the whole research field was 0.5 ha. The growing medium was sandy soil containing 1–3% of added peat.

Sampling. In October 1998, 15 randomly selected 7-year-old trees from each provenance (three trees from each block) were cut. The heights of trees including the five latest annual growths were measured. For chemical analysis, current-year needles from the middle of a lateral branch of the uppermost whorl were collected. From the main stem of the fourth annual growth, disks about 5 mm thick were cut for chemical analyses, for vertical stem area, and resin duct number measurements. The samples were stored in a cooling bag during sampling. The chemical samples were then stored in a deep-freezer (-80°C). The samples for microscopy were stored in a freezer (-20°C) until preparation and analysis. For chemical analyses, the whole stem disk without bark was used forming one sample.

Chemical Analyses. For terpene analysis, frozen needles and stem disks were cut into small pieces and extracted at room temperature with *n*-hexane for 1 hr. The hexane was filtered into screw-cap tubes, and the residue washed two times with *n*-hexane and filtered. The filtrates were pooled into one sample. 1-Chlorooctane was used as an internal standard. Resin acids were extracted from freeze-dried needles and stem disks using the method described by Gref and Ericsson (1985). Heptadecanoic acid was used as an internal standard. Terpene and resin acid samples were analyzed by gas chromatography–mass spectrometry (Hewlett Packard GC-type 6890, MSD type 5973) using a 30-m-long HP-5MS capillary column (ID 0.25 mm, film thickness 0.25 μm, Hewlett Packard). Helium was used as a carrier gas. The temperature program for terpenes was from 50°C to 250°C and for resin acids from 50°C to 270°C. The heating rate was 5°C/min. SCAN technique (mass numbers from m/z 30 to 350 were recorded; signal ions in monitoring; 93, 133, 136, 161, 204 m/z) was used for terpene samples, and the technique of selected ion monitoring (SIM) (299, 301, 314, 316 m/z) for resin acid samples. Only tricyclic resin acids were quantified. For identifying other possible resin acids in extracts, the SCAN technique was used in separate runs. Individual terpenes and resin acids were identified by their mass spectra and retention times. They were quantified by means of peak areas by using pure standards for calibration. The response factor for each compound was determined in relation to the known amount of internal standard. Korpilahti provenance trees were selected in order to compare the composition of mono- and sesquiterpenes between needles and stem disks. Some of these compounds were identified using available pure compounds. When pure compound was not available, identification was based on comparison of mass spectra with Wiley Library and Kovats retention indices (Davies, 1990; Elias et al., 1997). The proportional quantity of different compounds was calculated on the basis of the peak areas.

Growth Measurements and Vertical Resin Ducts. A stem cross-section area was measured in the fourth annual shoot of the trees. The stem disk without bark was scanned and the cross-sectional area was analyzed with an image analysis software (NIH IMAGE/Scion IMAGE). For light microscopic study, wood samples (1×1 cm) were cut from each stem disk and softened in boiling water for 1 hr. Wet samples were deep-frozen and sections approximately 16 μm thick were cut with a cryomicrotome. The sections were mounted on slides and stained with a mixture of safranin and Alcian blue (1:2). The number of vertical resin ducts in the radial wood was assessed with a light microscope (Zeiss) and the image analysis software (NIH IMAGE). The resin ducts were counted from two to three samples (image area approx. 2.37×1.77 mm^2) selected within the third and second growth rings of each cross section. Means per tree were used in statistical analyses.

Statistical Analyses. The data were analyzed with one-way analyses of variance (ANOVA), and the means were separated with the Tukey B procedure. An arc-sin transformation was used for percentage data, and some variables were $\log(n + 1)$ transformed to stabilize the variances before statistical analyses. Correlations were determined by using nonparametric Spearman correlation. All analyses were done with the SPSS statistical software package.

RESULTS

Comparison of Needle and Wood Chemical Composition. α-Pinene and 3-carene were the major monoterpenes, both in the needles and the wood (Table 1). They constituted about 67% of total monoterpenes in the needles and 74% in the wood. The wood showed lower quantities of tricyclene, camphene, and bornylacetate than the needles, while sabinene, limonene + β-phellandrene, and terpinolene were more abundant in the wood than in the needles (Table 1). The total monoterpene concentration was about five times higher in the needles than in the wood (Figure 1). Detailed terpene analyses of Korpilahti provenance trees showed that almost all identified monoterpenes were found both in the needles and wood (Table 2). *trans*-Ocimene and borneol were detected in the needles only. Overall, the number of sesquiterpenes was higher in the needles than in the wood (Table 2). The main sesquiterpene in the needles and wood was germacrene D. The other dominating sesquiterpenes were β-caryophyllene and α-muurolene in the wood, and germacrene B in the needles (Table 2).

A strong positive correlation was found between the proportional quantity of all terpenes, except myrcene, in needles and in wood (Table 3). If the proportional quantity of 3-carene in the needles or wood was high, the proportional quantities of sabinene and terpinolene were simultaneously high (Table 1). The concentrations of individual monoterpenes in the needles also positively correlated with corresponding wood values, but this relationship was weaker than observed with

TABLE 1. MEAN PROPORTIONAL QUANTITY (%) OF MONOTERPENES IN NEEDLES AND WOOD OF SCOTS PINE TREES REPRESENTING DIFFERENT SEED PROVENANCES[a]

Plant tissue and compound	Provenance: SAA South	RAK	TEN	RUO →	KOR	KIN	SUO	YLI	MUO North	*F*	*P*
Needles											
Tricyclene	2.0	1.9	1.9	2.3	2.2	2.0	2.2	2.2	2.1	0.924	0.499
α-Pinene	45.3	56.9	48.2	48.5	51.7	49.7	55.7	56.3	56.4	1.687	0.108
Camphene	8.5	7.9	8.0	9.5	8.7	8.1	8.8	8.9	8.3	0.655	0.730
Sabinene	1.5	1.0	1.4	1.4	1.2	1.3	1.1	1.1	0.9	2.590	0.012
β-Pinene	3.2	4.2	3.2	3.4	3.5	3.6	4.1	4.3	4.4	1.118	0.356
Myrcene	3.2	2.9	2.9	3.2	3.2	3.0	3.0	3.0	2.8	0.958	0.472
3-Carene	24.8	13.0	21.6	17.8	16.8	16.9	12.9	11.6	9.5	2.821	0.007
Limonene + β-phellandrene[b]	2.4	2.8	2.2	2.8	2.5	2.5	3.1	3.2	3.2	1.462	0.178
Terpinolene	2.5	1.4	2.3	2.0	2.0	1.9	1.5	1.8	1.2	2.072	0.043
Bornylacetate	6.7	8.0	8.1	9.3	8.1	11.0	7.6	7.7	11.3	1.094	0.372
Wood											
Tricyclene	0.1	0.2	0.1	0.2	0.2	0.2	0.1	0.2	0.2	2.224	0.030
α-Pinene	28.9	46.0	34.4	41.3	42.2	41.5	41.3	47.3	50.5	2.038	0.047
Camphene	0.5	1.0	0.6	0.9	0.8	0.8	0.8	0.9	1.1	2.287	0.025
Sabinene	3.7	2.5	3.0	2.4	2.1	2.4	2.4	1.7	1.5	3.051	0.004
β-Pinene	3.0	5.9	3.2	3.1	2.9	4.0	2.9	3.8	4.8	1.180	0.316
Myrcene	3.1	4.5	3.5	3.7	3.8	3.3	3.5	3.8	3.5	1.126	0.351
3-Carene	49.0	27.0	43.3	31.4	32.0	33.2	32.5	23.8	19.4	3.291	0.002
Limonene + β-phellandrene[b]	4.0	7.9	5.5	12.3	11.8	9.6	11.4	15.0	15.6	4.661	<0.001
Terpinolene	7.7	5.0	6.2	4.7	4.3	4.8	4.8	3.4	3.1	3.527	0.001
Bornylacetate	0.1	0.1	0.1	0.2	0.1	0.2	0.1	0.1	0.3	2.499	0.015

[a] SAA = Saaremaa, RAK = Rakvere, TEN = Tenhola, RUO = Ruokolahti, KOR = Korpilahti, KIN = Kinnula, SUO = Suomussalmi, YLI = Ylitornio, MUO = Muonio ($N = 15$). *F* (variance ratio) and *P* (significance) values from one-way analysis of variance are included. The provenances are ordered according the geographical distribution.

[b] Limonene and β-phellandrene were not separated from each other on HP-5MS column.

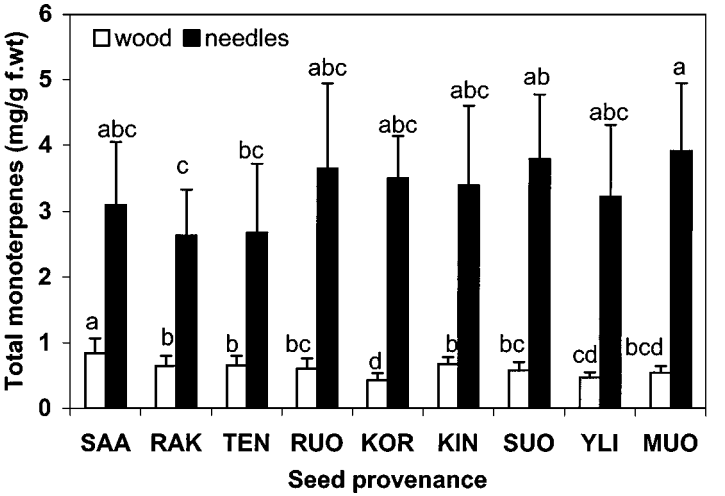


FIG. 1. Total monoterpene concentration (+SD) in wood and needles of Scots pine from nine different provenances growing in Suonenjoki nursery garden. Means followed by different letters are significantly different according to Tukey B procedure. Provenance names as described in Table 1. $N = 15$.

the proportional quantities (Table 3). No correlation was found between the total monoterpene concentration in needles and wood.

Pinifolic acid was the main resin acid in the needles of almost all trees, and isoabienol appeared to be the other main resin acid in the needles of several trees in each provenance (data not shown). Manoyl oxide acid occurred as the main resin acid in the needles of six pine individuals (two trees from SAA, three trees from RAK, and one from RUO) (data not shown). These above-mentioned labdane-type compounds were not found in the wood at all. Tricyclic resin acids, palustric + levopimaric and abietic acid, constituted about 80% of total resin acids in the wood (Table 4). The other tricyclic resin acids were found only in low quantities. The main tricyclic resin acids in the needles, palustric + levopimaric, dehydroabietic, abietic, and neoabietic acids, constituted about 75% of total tricyclic resin acids (Table 4). The total tricyclic resin acid concentration was four times higher in the wood than in the needles (Figure 2). The proportional quantities of different resin acids in the needles were only weakly related to the corresponding wood values, while no relationship was found between the resin acid concentrations of these different conifer tissues (Table 3).

Effects of Provenance on Needle and Wood Chemical Composition. The total monoterpene concentration in the needles or wood was highest in the northernmost MUO provenance and in the southernmost SAA provenance trees, respectively (Figure 1). In the needles, α-pinene concentration was lowest in TEN and

TABLE 2. PROPORTIONAL QUANTITY (%) OF MONO- AND SESQUITERPENES IN NEEDLES AND WOOD OF PINE INDIVIDUALS IN KORPILAHTI PROVENANCE ($N = 15$)

Compound group/compound	RRT[a]	Needles	Wood
Monoterpenes			
Tricyclene	0.627	1.5	0.2
α-Thujene[b]	0.641	0.1	0.1
α-Pinene	0.659	37.3	44.1
Camphene	0.695	6.3	0.7
Sabinene	0.760	0.7	1.4
β-Pinene	0.768	2.7	2.6
Myrcene	0.808	1.9	2.7
α-Phellandrene[b]	0.845	tr	0.1
3-Carene	0.859	10.7	25.6
α-Terpinene	0.876	0.1	0.4
Limonene + β-phellandrene	0.910	1.1	6.4
trans-Ocimene[b]	0.965	1.0	—
γ-Terpinene	0.994	0.1	0.3
unknown $C_{10}H_{16}$	1.069	—	0.2
Terpinolene	1.076	1.4	4.2
Borneol	1.296	0.3	—
Bornylacetate	1.617	6.4	0.1
unknown $C_{10}H_{16}$	1.740	1.2	0.1
unknown $C_{10}H_{16}$	1.746	—	0.2
unknown $C_{10}H_{16}$	1.770	0.3	0.6
Sesquiterpenes			
α-Longipinene[b]	1.775	—	0.2
α-Copaene	1.847	0.1	0.2
β-Elemene[b]	1.889	3.9	0.4
β-Caryophyllene	1.957	4.4	0.8
Calarene[b]	1.980	0.2	0.2
α-Humulene	2.037	0.8	0.1
unknown $C_{15}H_{24}$	2.093	0.3	0.1
Germacrene D[b]	2.105	7.0	2.0
β-Selinene[b]	2.117	0.2	0.1
Germacrene B[b]	2.139	4.5	0.7
α-Muurolene[b]	2.146	0.3	1.2
unknown $C_{15}H_{24}$	2.157	0.4	—
γ-Cadinene[b]	2.173	1.4	—
δ-Cadinene[b]	2.188	2.1	0.4
unknown $C_{15}H_{24}$	2.212	0.1	—
unknown $C_{15}H_{24}$	2.342	0.5	—
unknown $C_{15}H_{24}$	2.347	tr	—
unknown $C_{15}H_{24}$	2.356	0.4	—

[a] RRT, relative retention time.—, not found. tr, trace compound (<0.1%).
[b] Identified by comparison to Wiley Library and by comparison of Kovats retention indices based on authentic samples in the literature.

TABLE 3. SPEARMAN CORRELATION COEFFICIENTS OF PROPORTIONAL QUANTITY (%) AND ABSOLUTE CONCENTRATION OF MONOTERPENES AND RESIN ACIDS BETWEEN NEEDLES AND WOOD ($N = 135$)[a]

Compound	Correlation coefficient (%)	Correlation coefficient concentration
Monoterpenes		
Tricyclene	0.294**	0.078
α-Pinene	0.605**	0.253**
Camphene	0.285**	0.112
Sabinene	0.747**	0.593**
β-Pinene	0.458**	0.194*
Myrcene	0.162	−0.135
3-Carene	0.830**	0.682**
Limonene + β-phellandrene	0.465**	0.351**
Terpinolene	0.782**	0.634**
Bornylacetate	0.286**	0.231**
Resin acids		
Pimaric	0.221*	0.040
Sandaracopimaric	0.045	−0.084
Isopimaric	0.169*	0.090
Palustric + levopimaric	0.153	0.018
Dehydroabietic	0.250**	−0.118
Abietic	0.114	−0.095
Neoabietic	0.130	−0.025

[a] ** $P < 0.01$; * $P < 0.05$.

highest in MUO provenance trees, but its proportional quantity was similar in all provenances (Table 1). Although α-pinene concentration in the wood was at the same level in all nine provenances, its proportional quantity was higher in the MUO provenance trees compared to the SAA trees (Table 1). Actually, the proportional quantity of α-pinene in wood showed a clear increasing latitudinal gradient from south to north (Figure 3). The proportional quantity of 3-carene, both in needles and wood, was higher in SAA trees than in the northern MUO and YLI trees (Table 1), and in the case of wood samples, a clear decreasing latitudinal gradient from south to north was found (Figure 3). All trees from southern SAA and TEN provenances were high 3-carene ($\geq$10%) genotypes, and this genotype was rare among the northern provenance trees (Figure 4). Both the concentration and proportional quantity of limonene + β-phellandrene in wood (Table 1) were highest in the northern and central provenance trees, but terpinolene appeared to be more common in the southern provenance trees (Table 1). The quantity of camphene was higher in MUO than in SAA provenance trees.

The needles of trees from various Scots pine provenances contained a similar amount of total tricyclic resin acids (Figure 2). Abietic, levopimaric + palustric,

TABLE 4. MEAN PROPORTIONAL QUANTITY (%) OF RESIN ACIDS IN NEEDLES AND WOOD OF SCOTS PINE TREES REPRESENTING DIFFERENT SEED PROVENANCE[a]

	Provenance										
Plant tissue and compound	SAA South	RAK	TEN	RUO	KOR	KIN →	SUO	YLI	MUO North	*F*	*P*
Needles											
Pimaric	0.3	0.5	1.0	0.4	0.4	0.6	0.5	0.2	0.3	0.877	0.538
Sandaracopimaric	7.1	6.0	6.7	6.0	8.1	7.1	6.5	6.8	5.7	0.734	0.661
Isopimaric	1.3	1.8	0.9	0.9	0.9	0.7	1.8	0.9	1.4	1.530	0.153
Palustric + levopimaric	16.9	17.8	17.4	18.6	17.3	18.0	19.9	18.1	18.7	0.323	0.956
Dehydroabietic	23.7	21.0	20.8	18.4	21.6	19.9	17.6	20.1	17.0	0.762	0.637
Abietic	23.6	25.0	25.3	24.4	27.1	27.8	26.3	24.6	25.6	1.452	0.181
Neoabietic	27.1	27.9	27.9	31.3	24.6	25.9	27.4	29.3	31.3	0.722	0.672
Wood											
Pimaric	3.6	3.6	3.2	3.8	3.6	3.4	3.6	3.9	4.0	1.156	0.331
Sandaracopimaric	1.5	1.5	1.4	1.4	1.4	1.3	1.4	1.5	1.5	3.769	0.001
Isopimaric	1.1	1.3	1.1	0.9	1.0	0.9	1.0	1.2	1.3	0.647	0.737
Palustric + levopimaric	36.5	37.7	37.6	37.3	36.4	35.9	35.8	39.8	39.8	1.652	0.117
Dehydroabietic	2.3	3.0	3.2	2.7	2.6	2.6	2.7	3.2	2.9	1.492	0.166
Abietic	43.2	40.4	42.2	42.1	42.4	44.0	43.6	38.3	36.8	2.527	0.014
Neoabietic	11.8	12.5	11.3	11.8	12.6	11.9	11.9	12.1	13.7	2.710	0.009

[a] SAA = Saaremaa, RAK = Rakvere, TEN = Tenhola, RUO = Ruokolahti, KOR = Korpilahti, KIN = Kinnula, SUO = Suomussalmi, YLI = Ylitornio, MUO = Muonio ($N = 15$). *F* (variance ratio) and *P* (significance) values from one-way analysis of variance are included. The provenances are ordered according the geographical distribution.

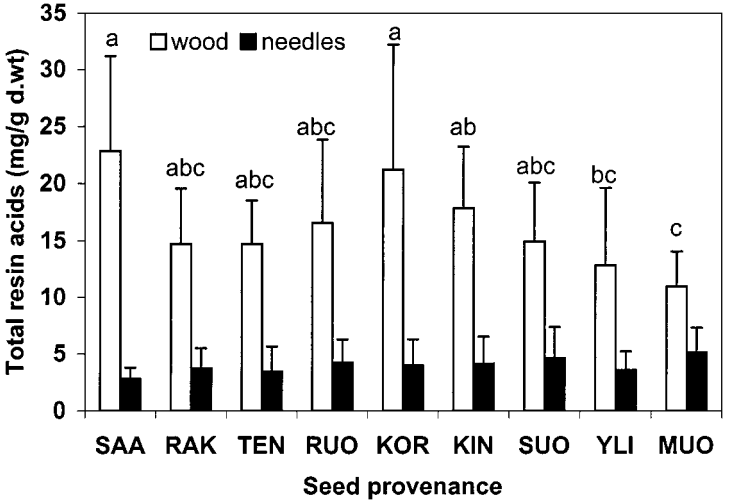


FIG. 2. Total resin acid concentration (+SD) in wood and needles of Scots pine from nine different provenances growing in Suonenjoki nursery garden. Means followed by different letters are significantly different according to Tukey B procedure. Provenance names as described in Table 1. $N = 15$.

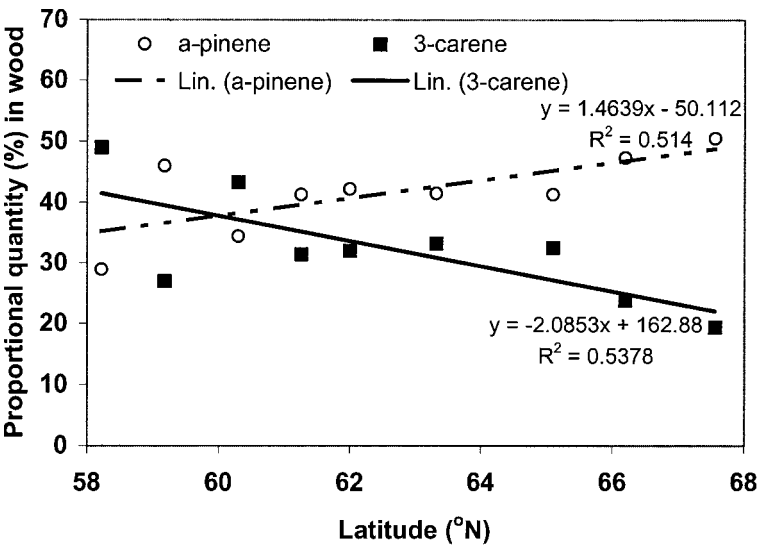


FIG. 3. Mean proportional quantity of α-pinene and 3-carene in Scots pine wood as a latitudinal gradient. Symbols represent results of different provenances at different latitudes. Linear relationship between the latitude and the proportional quantity of each compound is given.

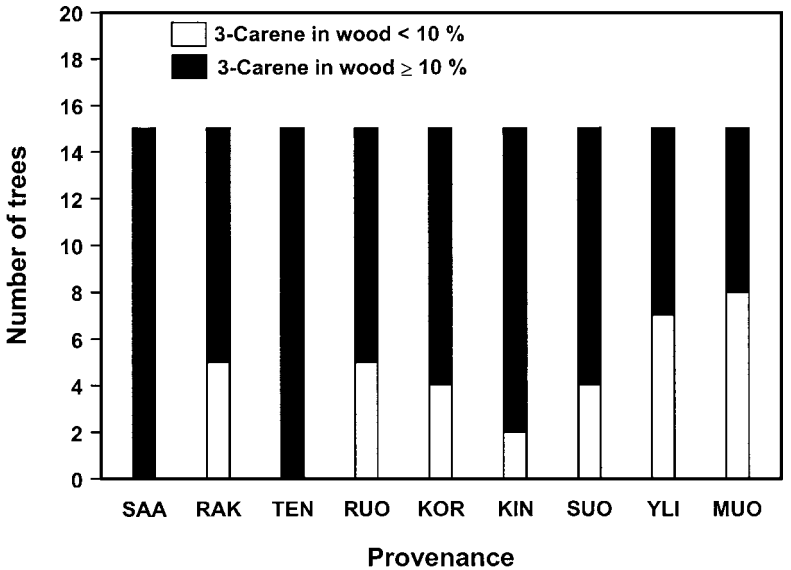


FIG. 4. The number of Scots pine trees with a low 3-carene proportion in wood (open part of bar) or with a high 3-carene proportion in wood (solid part of bar) in Scots pine from nine different provenances growing in Suonenjoki nursery garden. Provenance names as described in Table 1.

and neoabietic acid concentrations were lower in YLI provenance trees than in the other northern provenance trees. In contrast, the wood of MUO provenance trees showed significantly lower total resin acid concentration than the wood of SAA and KOR provenance trees (Figure 2). All individual resin acid concentrations in the wood, except isopimaric acid, were significantly affected by provenance. The trees from the southernmost SAA provenance and from central KOR provenance showed the highest abietic and levopimaric + palustric acid concentrations. The proportional quantities of different resin acids in the needles were similar in all the provenances (Table 4). In the wood, however, the proportional quantity of abietic acid was significantly higher in KIN and SUO provenance trees than in MUO provenance trees (Table 4).

Growth Measurements and Resin Ducts. The trees from KOR provenance showed the best growth in height of all provenances (data not shown), being significantly taller than trees from SAA, RAK, KIN, and YLI provenances. Pine trees originating from SAA had the smallest radial growth of stem. RUO and KOR trees had the best radial growth, but fewer vertical resin ducts per 1 mm^2 wood than pines from KIN provenance. The total monoterpene concentration in the wood positively correlated with the vertical resin duct number ($r = 0.188$, $P < 0.05$, $N = 132$) and negatively with the radial growth of stem ($r = -0.395$, $P < 0.001$,

$N = 132$). Tricyclic resin acid concentration in the wood did not correlate with the vertical resin duct number or with the radial growth of stem.

DISCUSSION

Needle and Wood Chemical Composition. Monoterpene composition in Scots pine needles and wood appeared to be fairly analogous, α-pinene and 3-carene being the major monoterpenes in both tissues. The monoterpene composition of needles was in accordance with many earlier Scots pine studies (Hiltunen et al., 1975; Chalchat et al., 1985; Muona et al., 1986; Sjödin et al., 1996). The monoterpene composition of wood has not been thoroughly studied so far, and comparisons between monoterpene compositions in needles and wood of Scots pine are rare (Sjödin et al., 1993, 1996, 2000). The proportional quantity of 3-carene in wood and in needles within same pine individuals was similar, which coincides with the findings of Sjödin et al. (1996). Thus, the present results suggest that needle analysis might be used to estimate the proportional quantity of 3-carene in the wood of Scots pine and vice versa. According to a correlation analyses, sabinene and terpinolene could also be used for this purpose. However, Latta et al. (2000) concluded that a high error is associated in that kind of prediction. In their study with Ponderosa pine, needles were the most variable tissue in monoterpene composition, and needle monoterpene composition weakly correlated with the composition of other tissues.

Proportional quantities of 3-carene, sabinene, and terpinolene were simultaneously high in both analyzed tissues. Similar results have been found earlier in cortical oleoresin studies (Yazdani et al., 1982; Baradat and Yazdani, 1988) leading to the suggestion that these three monoterpenes are formed by a common biosynthetic pathway. The low quantity of camphene in the wood agrees with the study of Sjödin et al. (1993). In low 3-carene trees ($<10\%$), α-pinene and limonene made up to 85% of total wood monoterpenes. This finding is also consistent with the earlier study of Sjödin et al. (1993).

Resin acid composition and concentration in Scots pine needles and wood were quite different, suggesting different biosynthetic mechanisms in the two tissues. Labdane-type pinifolic acid represented the major resin acid in most needle samples, and this is consistent with earlier studies (Gref and Tenow, 1987; Andersson et al., 1990; Danell et al., 1990). Another labdane-type resin acid, manoyl oxide acid, was infrequent in the needles [detected earlier with Scots pine trees growing in Scandinavia (Andersson et al., 1990) and elsewhere (Tobolski and Zinkel, 1982)]. In earlier studies, Scots pine seedlings originating from central Finland had abietic acid as the main tricyclic resin acid in the shoots (Manninen et al., 1998a) and needles (Kainulainen et al., 1996). In the present study, the same result was found with needles originating from KOR and KIN provenances.

In contrast, wood in the present study and Scots pine bark in the earlier studies (Tobolski and Zinkel, 1982; Gref and Ericsson, 1985) contained only tricyclic resin acids. Palustric + levopimaric acid made up to 35% of total resin acids in our study, which agrees with an earlier Scots pine study (Bridgen and Hanover, 1982). No strong correlation was detected between the proportional quantities of resin acids in needles and wood. Thus, it could be assumed that the resin acid composition of needles is not necessarily a good tool for estimating the corresponding composition in wood.

Effects of Provenance. Because all Scots pine seedlings were growing under similar environmental conditions in the Suonenjoki nursery, the observed differences in the needle and wood monoterpenes between provenances may mainly be due to genetical variation. Undisturbed growth of over five years in the nursery garden ensures that the effect of planting on terpenoids was minimized (Sallas et al., 1999). The variation of needle monoterpenes in Scots pines collected from different geographical locations in Finland has been evident in previous studies (Hiltunen et al., 1975; Muona et al., 1986). Individual trees in the present study could be characterized as high 3-carene trees ($\geq$10%) or low 3-carene trees ($<$10%) based on the limits set for needles (Hiltunen et al., 1975; Muona et al., 1986). The number of high 3-carene trees was smaller among the northern provenances than in southern ones. A similar north–south gradient of high 3-carene trees was found earlier with Scots pines growing in different parts of Finland (Hiltunen et al., 1975; Muona et al., 1986). Genetic isolation of the SAA and TEN provenances could explain why all trees from them were high 3-carene trees.

In the present study, the proportion of α-pinene in needles did not increase towards northern provenances, while shoots of the same provenances (Nerg et al., 1994) showed an increase. Higher total monoterpene concentration in the southern and coastal provenance trees than in the other provenance trees originating from more inland areas may indicate that the southern provenances represent a geographically isolated area. Geographical isolation of insular populations was also suggested to explain the distinctive low β-pinene concentrations in *Pinus nigra* in Western Europe (Rafii et al., 1996).

Muona et al. (1986) found that the proportional quantity of camphene in needles within the low 3-carene group in the natural stands was higher in northern Finland than in southern Finland. In our study, a similar trend was found in the wood samples only. In Sweden, in the most northern Scots pine populations, the majority of trees contained a high proportion of limonene in the cortex, while in southern populations a high proportion of sabinene was found (Yazdani and Nilsson, 1986). A similar north–south trend in the present wood results was found with limonene. Terpinolene in wood, together with 3-carene, had the highest concentrations in southern populations, suggesting that wood monoterpene composition is genetically controlled.

The total tricyclic resin acid concentration and the proportional quantities of individual tricyclic resin acids in Scots pine needles were independent of the provenance. This was also shown for shoots of the same pine provenances (Nerg et al., 1994). However, variations in resin acid composition in the needles and in the cortex between Scots pine provenances have been reported earlier (Tobolski and Zinkel, 1982). In our study, only the isopimaric acid concentration in needles was affected by provenance, consistent with the results of Hansson and Gref (1987). In contrast, wood resin acids showed more variation among provenances than resin acids in the needles. Thus, low resin acid concentration in the most northern MUO provenance may be genetically determined. Overall, among Scots pine varieties, Scandinavian ones had the highest total resin acid concentration in wood compared to other regions in Europe (Bridgen and Hanover, 1982).

The best growth of KOR provenance seedlings in the Suonenjoki location could be partly explained by the geographical proximity of the locations. In our long-term observations, trees from provenances closest to Suonenjoki, i.e., KIN, KOR, and RUO, have shown the best growth in Suonenjoki (Martti Vuorinen, personal communication). The differences in growth conditions, e.g., highest effective temperature sum in the original area of SAA provenance (Nerg et al., 1994), and differences in genetic factors may provide explanations for the smallest radial growth of stems originating from coastal SAA provenance. Although frost hardening is initiated according to the intrinsic attributes of genotypes, all provenances become hardy enough to tolerate the winter conditions in Suonenjoki (Repo et al., 2000).

The small number of vertical resin ducts in stems of KOR provenance may be associated with the fast growth of trees, when the same amount of resin ducts distributes over a larger area. This was also suggested by Blanche et al. (1992) with *Pinus taeda*. Another explanation for this finding may be the competition of resources between growth and differentiation (i.e., production of secondary metabolites) (Herms and Mattson, 1992). However, Bridgen and Hanover (1982) found no genetic variation in the resin duct density of Scots pines. In our study, monoterpene concentration in wood negatively correlated with radial growth of stem, but in maritime pine, the tree diameter showed no relation to the proportional quantity of different terpenes in wood (Kleinhentz et al., 1999). Wood resin acids did not correlate with the vertical resin duct density or growth of trees in our study, but resin flow of loblolly pine positively correlated with vertical duct density (Blanche et al., 1992). In addition, wood resin acids did not correlate with resin duct area in the study of Bridgen and Hanover (1982). However, Saikkonen et al. (1995) found a positive correlation between the total resin acid concentration and the proportional resin duct area of the needles. This could mean that resin acids in the wood are also located elsewhere than only in resin ducts. Horizontal resin ducts in wood were not analyzed in this study.

In conclusion, Scots pine needles and wood exhibit similar monoterpene composition. The proportion of 3-carene in needles especially may serve as an estimator for the 3-carene content in wood tissue and vice versa. Resin acid composition in the needles and wood is different, and resin acid concentration in the wood is much higher than in the needles. Needles are exposed more directly to, e.g., insect herbivores than wood and, thus, they may be constrained to produce a higher concentration of volatile monoterpenes than wood. Cortex and the cortical volatile oleoresin compounds are already a chemical barrier against external irritants threatening the wood. The effect of geographical provenance on needle and wood terpenoids is evident. Vertical resin duct numbers showed slight correlation with total monoterpene concentration in wood. Thus, the number of vertical resin ducts might not be useful tool to estimate the amount of oleoresin in wood tissue.

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WHY PHENOLIC ACIDS ARE UNLIKELY PRIMARY ALLELOCHEMICALS IN RICE

MARIA OLOFSDOTTER,* MALOU REBULANAN, ARTEMIO MADRID, WANG DALI, DOMINGO NAVAREZ, and DANIEL C. OLK

International Rice Research Institute
PO Box 3127, Makati Central Post Office
1271 Makati City, Philippines

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Abstract—Allelopathy in rice (*Oryza sativa*, L.) effective against weeds has been found in about 3.5% of tested rice germplasm in both laboratory and field experimentation. However, the allelochemicals responsible for growth inhibition of rice-associated weeds have not yet been identified. In the literature, phenolic acids are often mentioned as putative allelochemicals. If phenolic acids commonly reach growth inhibitory concentrations in rice ecosystems, it must be expected that the degree of tolerance to phenolic acids will vary among traditional rice cultivars or plant species adapted to rice environments having inherently different phenolic acid concentrations. Phenolic acids concentrations are normally greater in submerged than in aerobic soils. A dose–response study, however, showed that seedlings of rice cultivars adapted to submerged anaerobic soils did not have higher level of tolerance against *p*-hydroxybenzoic acid than did seedlings of varieties adapted to aerobic upland soils. Moreover, traditional rice cultivars had no greater tolerance than did improved cultivars that were recently bred for traits other than tolerance of phenolic acids. Similarly, there were no differences in tolerance of *p*-hydroxybenzoic acid between two *Echinochloa* weed species adapted to either anaerobic or aerobic growth conditions. Thus, neither the rice cultivars nor weed species had evolved different tolerance levels against the phenolic acid. However, all rice cultivars had significantly greater tolerance of *p*-hydroxybenzoic acid than did either weed species. In a second experiment, the rates at which rice plants released phenolic acids into solution cultures were measured for at least one month, the time period of greatest allelopathic activity following planting under field conditions. The maximum release rate of phenolic acids during the first month of growth was approximately 10 μg/plant/day. At a conventional plant density, the release rate of phenolic acids would be approximately 1 mg/m^2/day. This order of release rate cannot provide concentrations

*To whom correspondence should be addressed. Weed Science, Agrovej 10, 2630 Taastrup, Denmark.

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remotely close to phytotoxic levels determined for these rice cultivars and weed species. The results presented in this paper do not preclude the possibility that phenolic acids might be one component in a mixture of chemicals that, when present simultaneously, are allelopathic.

Key Words—Allelopathy, *Oryza sativa*, phenolic acids, phytotoxicity, rice, weed suppression.

INTRODUCTION

Allelopathy is the direct influence of a chemical released from one plant on the development and growth of other plants. Its potential as a natural means for weed suppression in agrosystems has long attracted interest. Allelopathy in rice effective against weeds has been found in about 3.5% of tested rice germplasm in both laboratory (Fujii, 1992; Navarez and Olofsdotter, 1996) and field experimentation (Dilday et al., 1998; Olofsdotter et al., 1999). Under field conditions, in three different growing seasons, some strongly allelopathic cultivars were capable of reducing *Echinochloa crus-galli* L. biomass by more than 50% compared to non-allelopathic rice cultivars (Olofsdotter et al., 1999). This biomass reduction was expressed as reduced biomass per plant, whereas germination and total number of plants were unaffected. Despite realized effects in the field, the allelochemicals responsible for growth inhibition of rice-associated weeds have not yet been identified.

In the literature on allelopathy, phenolic acids are often mentioned as putative allelochemicals and are perhaps the most commonly investigated compounds among potential allelochemicals. They are found in a wide range of soils, and their phytotoxic potential against various plants has been demonstrated under controlled conditions at concentrations greater than about 1 mM (Hartley and Whitehead, 1985; Dalton, 1999). Studies of mixtures of phenolic compounds, however, have shown that individual concentrations can be additive when evaluating for phytotoxic affects (Blum, 1996). This means that concentrations under 1 mM can become important when in mixture with other phenolic acids.

Phenolic acids are known root exudates of rice, and Mattice et al. (1998), suggested that allelopathy of rice against weeds was correlated with the amounts of phenolic acids released by living rice roots. Their work showed that rice cultivars that were allelopathic against ducksalad (*Heteranthea limosa*) released increased amounts of several isomers of hydroxybenzoic acid (HBA) and two isomers of hydroxyphenylpropanoic acid compared with nonallelopathic rice cultivars (Mattice et al., 1998). Phenolic acids have also been suggested to cause autotoxicity problems in double-cropped rice systems. Chou et al. (1977) attributed inhibited germination and poor early season growth of a second rice crop in Taiwan to phenolic acids arising from decomposing residues of a first rice crop. Alternatively, however, poor early season rice growth has also been associated with inhibition

of nitrogen (N) uptake, due either to the presence of aromatic acids such as 2-phenylpropanoic acid, 3-phenylpropanoic acid, and benzoic acid, all microbial metabolites in soil under highly reduced conditions (Tanaka and Nishida, 1998), or to biological N immobilization (Niranjan Rao and Mikkelsen, 1976; Witt et al., 2000).

Allelochemicals are often identified by testing for phytotoxicity under controlled conditions of a given chemical found in the plant or associated soil. Such studies need to consider not only the chemical composition sensu stricto but also the mixture of compounds, the dose, and the duration the plants experience under a given environment. In rice soils, for example, Kuwatsuka and Shindo (1973) isolated 13 different phenolic acids during decomposition of rice straw. They showed that *p*-coumaric acid was released in the highest amount. However, over time, during decomposition, release of all phenolic acids except *p*-HBA decreased considerably. The importance of these phenolics as allelochemicals in the rice ecosystem must be questioned, because the concentrations needed for phytotoxicity, as determined by Chou and Chiou (1979), far exceeded the concentrations commonly found in natural conditions (Tsutsuki and Ponnamperuma, 1987).

In another study, Hsu et al. (1989) evaluated the concentrations of phenolics needed to inhibit germination of lettuce (*Lactuca sativa*) and alfalfa (*Medicago sativa*). From their data, we calculated the ED_{50g}, which is the effective dose of phenolic acids required to inhibit germination of lettuce and alfalfa by 50%, respectively (Table 1). Salicylic and *p*-HBA were the strongest germination inhibitors but only at very high concentrations (200–350 mg/kg solution). Their data also allowed calculation of the time needed to reach 50% germination, which was lengthened by most phenolic acids tested (Table 1).

In fact, a phenolic acid typically has a concentration less than 5 mg/kg soil in submerged rice soils without amended rice straw. Amendment of rice straw (5 tons/ha) has been shown to raise the average level of phenolic acids seven-fold (Tsutsuki and Ponnamperuma, 1987), which would still be well below phytotoxic levels. However, soil is not a uniform medium, and there may be pockets with concentrations substantially higher than the bulk mean concentration (Foy, 1999; Dalton, 1999), such as the rhizosphere of living plants and the immediate vicinity of degrading plant tissues. In summary, additional evidence is necessary before phenolic acids can be considered as primary allelochemicals in rice.

Rice has been grown for several thousand years, and the evolution of traditional cultivars has been driven by local adaptation for site-specific climatic conditions, pest tolerance, and higher yield. As such, traditional rice varieties are expected to be adapted to the most important features in their growing environment. Therefore, it must be expected that traditional plant cultivars or plant species adapted to environments with inherently different phenolic acid concentrations would vary in their tolerance of phenolic acids if phenolic acid content indeed reaches growth inhibitory concentrations in parts of the soil. Similarly, the

TABLE 1. INHIBITION AND DELAY OF LETTUCE AND ALFALFA GERMINATION BY PHENOLIC ACIDS[a]

	Lettuce (*Lactuca sativa*)		Alfalfa (*Medicago sativa*)	
Phenolic acid	$ED_{50\,g}$ (mg/kg)[b]	Δ(t) (hr)[c]	$ED_{50\,g}$ (mg/kg)[b]	Δ(t) (hr)[c]
Ferulic	≅700	23	≫1000	5
Vanillic	≅400	26	>1000	13
Caffeic	>1000	<5	≫1000	<5
p-Coumaric	≅550	≅10	>1000	10
p-Hydroxybenzoic	≅300	16	≫1000	6
Salicylic	<200	-	≅350	≅10
α-Resorcylic	≅650	10	≫1000	<5
Gallic	>1000	20	>1000	≅5

[a] Only phenolic acids present in rice soils are included. Recalculated from Hsu et al. (1989).
[b] concentration needed to inhibit germination by 50%. Units are mg kg^{-1} solution.
[c] the difference in time to reach 50% germination.

capacity of weeds to develop specific adaptations to biochemical controls is well documented (Jordan and Jannink, 1997), and some insects have developed tolerance toward or have even acquired the capability to benefit from phenolic-rich substrates (Appel, 1993). In a second comparison among rice cultivars, modern varieties, derived from traditional cultivars and bred for higher yield, might be expected to have a lesser degree of adaptation to environmental stresses than the traditional cultivars, as plant characteristics not included in the selection procedure are in principle selected against (G. S. Khush, personal communication).

Under irrigated rice (anaerobic) environments, the average content of phenolic acids in the soil, at least during early crop growth stages, is considerably larger than under upland (aerobic) conditions because of the slower degradation of phenolic acids under anaerobic conditions (Shindo and Kuwatsuka, 1977; Hartley and Whitehead, 1985). It is not clear, however, whether phenolic acid concentrations are maintained at higher levels in lowland soils than in upland soils over longer periods of time. Leaching losses might be greater in lowland soils (Shindo et al., 1979), and the soil temperature can be higher in lowland soils, which would accelerate decomposition (Chou et al., 1981). Anaerobic microbial degradation of phenolic acids may also be delayed by reversible sorption of the phenolic acids to the soil (Blum, 1998). Such sorption, however, will also influence the magnitude and duration of phytotoxicity for these compounds (Cheng, 1995; Blum, 1998). Sorption of phenolic acids to soil, however, could occur in either situation in both lowland and upland soils and cannot explain differences between upland vs lowland.

If naturally occurring concentrations of phenolic acids are inhibiting growth and if plants have adapted to their environmental conditions, plants normally growing under anaerobic conditions should have a higher level of tolerance towards phenolic acids than plants adapted to aerobic conditions, and the degree of adaptation by lowland rice should be greater in traditional varieties than in modern varieties since it has not been a primary breeding objective.

While knowledge of interactions among phenolic compounds has gradually accumulated in aerated soils, virtually nothing has been done on phenolic compounds as allelopathic agents in irrigated rice soils. Knowledge gained in aerated soil is not always directly transferable to irrigated rice. The rice plant has different root processes than most other plants, and the anaerobic soil conditions also provide for potentially different biological and chemical environments. Hence, this study was set up under controlled conditions, first, to establish any differences in tolerance for *p*-hydroxybenzoic acid among weed species, traditional rice, and modern rice cultivars adapted to irrigated lowland or upland conditions. We selected *p*-hydroxybenzoic acid to represent phenolic acids, based on the phytotoxicity results of Hsu et al. (1989), the increased release of HBA in allelopathic rice (Mattice et al., 1998), the demonstrated phytotoxic nature of this acid when tested on a range of weeds (Reigosa et al., 1999), and the widespread belief that this particular phenolic acid is often involved in host plant resistance to weeds, pests, and diseases (Niemeyer, 1988). As a second objective, the amounts of phenolic acids released from living rice plants, allelopathic or not, were measured and compared with the tolerance levels of *p*-hydroxybenzoic acid in order to evaluate whether the amounts of naturally exuded *p*-hydroxybenzoic acid sufficed for allelopathic activity of rice, assuming additive effects of phenolic acids in mixture (Blum, 1996; Reigosa et al., 1999).

METHODS AND MATERIALS

Dose–Response Curves for p-Hydroxybenzoic Acid. Four rice cultivars and two weed species were chosen to represent cultivars and species adapted to either irrigated (anaerobic) or rainfed upland (aerobic) environments. The upland rice cultivars were Moroberekan and IRAT 216, representing traditional and modern (improved) japonicas, respectively. Cultivars adapted to irrigated and anaerobic conditions were represented by Mahsuri (traditional) and IR5 (improved), respectively, both indicas. *Echinochloa crus-galli* and *Echinochloa colona*, both of which can be suppressed by allelopathic rice cultivars (Olofsdotter et al., 1999; Pheng et al., 1999), were chosen as the weeds adapted to anaerobic and aerobic conditions, respectively. Seeds for all rice cultivars and both weed species were obtained from field soils maintained under representative growing conditions.

All seeds (20 per Petri dish) were sown on filter paper in a Petri dish placed inside a small growing box, closed with a removable plastic cover. A filter paper strip formed a liquid bridge from the surrounding box into the Petri dish. By this technique, an excess of test solution can be added to the box to ensure a sufficient amount of water to the test plants without drowning the seeds. Thirty milliliters of test solution was added to the surrounding box, outside the Petri dish, at the start of the experiment and 20 ml was added at day 4. Seven different concentrations of *p*-hydroxybenzoic acid were applied as the test solution in the plant box. Concentrations were chosen to cover the entire range of responses from virtually unaffected to severe inhibition or death. When log-transformed, concentrations were evenly spaced along the dose–response curve. Two weeks after seeding the plants were harvested, shoot and root length were measured for all germinating plants, and shoot and root dry matter were measured for each Petri dish. Germination of individual seeds was recorded from 2 days after seeding until all seeds had germinated. The experiments were replicated five times and repeated once over time.

Statistics. Plant growth (y) in response to a dose (z) can be described by a four-parameter logistic model (Streibig, 1988):

$$y = (D - C)/1 + \exp\left\{2b * \left[\log(\mathrm{ED}_{50}) - \log(z)\right]\right\} \quad (1)$$

where D denotes the upper and C the lower limit of response at low and high doses, respectively. ED_{50} defines the dose required to decrease the response by 50% relative to untreated and is equal to a response $(D + C)/2$. Parameter b is proportional to the slope of the curve at ED_{50}. If a phytotoxin has the same mode of action regardless of cultivar or weed, the dose–response curves would theoretically have the same slope. Such parallelism can be statistically tested by the F test.

Variation in this kind of experiment is rarely normally distributed. Therefore, data are typically transformed to provide a normal distribution. A suitable family $h(\lambda)$ of transformations was found by Box-Cox analyses (Box and Cox, 1964), and a transform-both-sides technique was used (Carroll and Ruppert, 1988). All statistical procedures were done in SAS.

Release Rates of Phenolic Acids from Living Rice Plants. Nine rice cultivars were selected for this experiment based on our previous rice allelopathy study (Olofsdotter and Navarez, 1996). Four nonallelopathic rice cultivars were selected: AUS196 (improved AUS), IR38 (improved indica), Mahsuri (traditional indica), Brown Gora, (traditional indica), all lowland rice. Five allelopathic cultivars were selected: Woo Co Chin Yu (traditional indica), IR64 (improved indica), Taichung Native1 (traditional indica), AC1423 (improved indica), and IR56 (improved indica), all lowland rice. The five allelopathic cultivars had different strengths of allelopathic activity toward weeds, and their correlation with the rate of phenolic release would test the significance of phenolics in weed-suppressing

allelopathy. Rate of phenolics release was monitored in four cultivars until 56 days after transplanting, and in another experiment the remaining five cultivars plus IR38 (control) were measured only until 28 days after transplanting, mirroring the most important time period of allelopathic weed control. The plants were grown in hydroponic solutions in the greenhouse, and the experiment was carried out in a randomized complete design with three blocks and repeated once over time.

Rice seeds were surface-sterilized with 2.6% sodium hypochloride for 20 min, rinsed three times with distilled water, and germinated in Petri dishes at room temperature. Twelve days after seeding, seedlings with uniform root length and shoot height were wrapped with foam, inserted into holes in a styrofoam float, and transplanted into the hydroponic solutions. Two seedlings were grown 10 cm apart in a 3.6-liter pail filled with hydroponic solution. The pails were wrapped with aluminum foil to inhibit algae growth and placed in a bench with cool water to avoid overheating. The nutrient/hydroponic solution (Yoshida et al., 1976) was changed every 2.5 days to ensure sufficient nutrients to the rice plants, and its pH was maintained at 5.5 daily by using 0.1 N NaOH throughout the experiment. The nutrient/hydroponic solution was kept at half strength for the first week after transplanting, and thereafter full-strength solution was used.

The hydroponic solution was first sampled two weeks after transplanting (WAT). Thereafter the solution was sampled weekly. Twenty-four hours before every sampling, the nutrient solution was replaced with 3.6 liters of solution with the same N concentration as before but in the form of NH_4Cl instead of NH_4NO_3 to avoid possible interference by nitrate in the determination of total phenolic compounds. At sampling, the plants were lifted out of the hydroponic solution, the solution stirred, and 500 ml removed for analysis. The release rates of phenolic compounds were determined through the amount of detected phenolics released by the plants during the previous 24 hr. The sample was filtered through Whatman No. 1 filter paper and analyzed for total phenols by using the 4-aminoantipyrine colorimetric method (Clesceri et al., 1989) with *n*-phenol (C_6H_5OH) as the standard. This method covers the phenolic compound range from 1 to 250 μg/liter with a sensitivity of 1 μg/liter. The minimum detectable quantity is 0.5 μg/liter. Obtained values represent a minimum amount of phenolic compounds present in the sample, as the reactivity with 4-aminoantipyrine varies by phenolic compound. Typical standard deviations for such an analysis are 12%, which should be considered when interpreting the data obtained (Clesceri et al., 1989).

Release rates of total phenolic compounds per day per plant were calculated as:

$$\mu\text{g phenol/plant} * \text{day} = (A/B \times 1000 * 3.6)/2 \qquad (2)$$

where A is the amount phenol measured per microgram of sample as determined from a calibration curve and B is the volume of the sample.

TABLE 2. PARAMETERS FOR NORMALIZED *p*-HYDROXYBENZOIC ACID DOSE–RESPONSE CURVES FOR ROOT LENGTH OF FOUR RICE CULTIVARS AND TWO WEED SPECIES

	D	−*b*	ED_{50}	*C*
Moroberekan	102	1.77	653	−1.1
	(86–118)	(0.95–2.60)	(540–767)	(−5.6–3.3)
Mahsuri	101	2.53	639	1.3
	(90–112)	(1.69–3.38)	(571–708)	(−0.5–3.2)
IR5	99	2.44	528	8.8
	(89–109)	(1.17–3.52)	(461–595)	(3.1–14.4)
IRAT 216	101	2.54	678	−0.3
	(90–113)	(1.64–3.44)	(600–756)	(−1.7–1.0)
Echinochloa crus-galli	101	1.53	334	0.4
	(80–121)	(1.09–1.97)	(264–409)	(−0.8–1.6)
Echinochloa colona	101	2.05	310	0.1
	(74–128)	(1039–2.72)	(241–380)	(−0.3–0.6)

[a] *D* denotes the upper and *C* the lower limit of response at low and high doses, respectively. ED_{50} is the dose required to decrease the response by 50% and is equal to a response $(D + C)/2$. The parameter *b* is proportional to the slope of the curve at ED_{50}. Confidence limits (0.05) for the estimated parameters are given in parentheses.

RESULTS

Dose–Response Curve for p-*Hydroxybenzoic Acid.* The dose–response equation (1) adequately described the dose–response relationship for root length of all rice cultivars and both weed species. The estimated parameters from normalized dose–response curves did not vary among rice cultivars (Table 2). Rice cultivars adapted to submerged anaerobic conditions did not have a higher level of tolerance against *p*-hydroxybenzoic acid than did upland varieties, and traditional varieties had no greater tolerance than did improved varieties. Similarly, there were no differences in tolerance of *p*-hydroxybenzoic acid between the two weed species adapted to either anaerobic or aerobic growth conditions (Table 2). This means that, parallel to the findings in rice, weed species have not evolved tolerance against the phenolic acid that reflects their growth environments.

When comparing all rice cultivars with both weeds, there was a significant difference in their tolerance of *p*-hydroxybenzoic acid. The two *Echinochloa* species had ED_{50} values only half of those of the rice cultivars (Table 2). Consequently *p*-hydroxybenzoic acid can selectively control the *Echinochloa* species at a concentration that does not affect rice. The slopes of the dose–response curves at ED_{50} (*b* parameter) did not differ significantly between all rice cultivars and the weeds, indicating a similar mode of action for growth inhibition both in rice and weeds. Root dry weight showed the same trends as did root length, and shoot length and shoot dry weight had smaller but similar levels of response (data not shown).

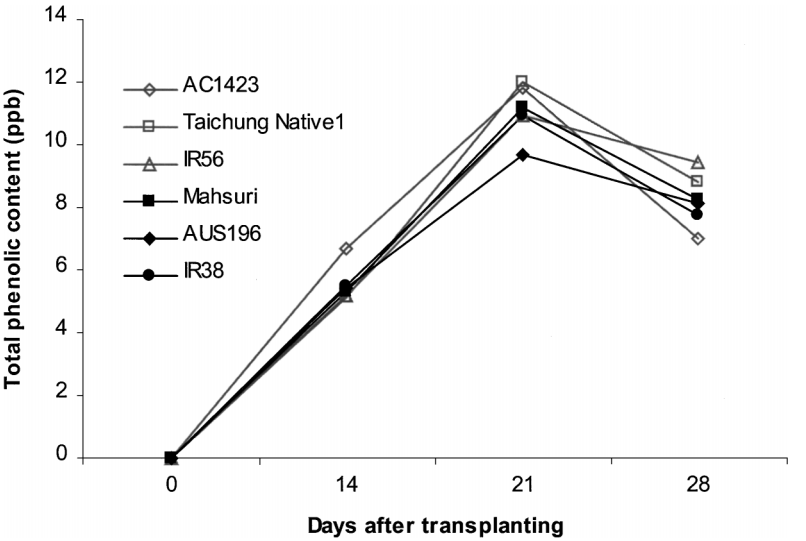


FIG. 1. Total phenolic acids released (μg/plant/day) for three allelopathic rice cultivars (open symbols) and three nonallelopathic rice cultivars (closed symbols) into hydroponic solution.

Release Rates of Phenolic Acids from Living Rice Plants. Allelopathic rice cultivars did not release higher amounts of phenolic acids than nonallelopathic cultivars (Figure 1). Similarly there were no differences in the amounts released from traditional rice cultivars compared to improved rice cultivars (Figure 2). All rice cultivars had an increasing release rate of phenolic acids until at least 14 days after transplanting, whereafter a slight decrease in the amount released occurred at 28 days after transplanting. For the cultivars that were studied until 56 days after transplanting, the amounts released increased after 28 days after transplanting.

The maximum release rate of phenolic acids from rice plants during the first month of growth, when allelopathy normally affects weed growth, was approximately 10 μg/plant/day (Figures 1 and 2). At a conventional plant density of 100 rice plants/m^2, the release rate of phenolic acids would be approximately 1 mg/m^2/day. Even if all phenolic acids were as phytotoxic as *p*-hydroxybenzoic acid, this release rate could not provide concentrations within one month remotely close to phytotoxic levels for the rice cultivars studied here (Table 2).

DISCUSSION

Dose–response relations with *p*-hydroxybenzoic acid presented in this paper agree with other studies on the magnitude of phytotoxicity of phenolic compounds

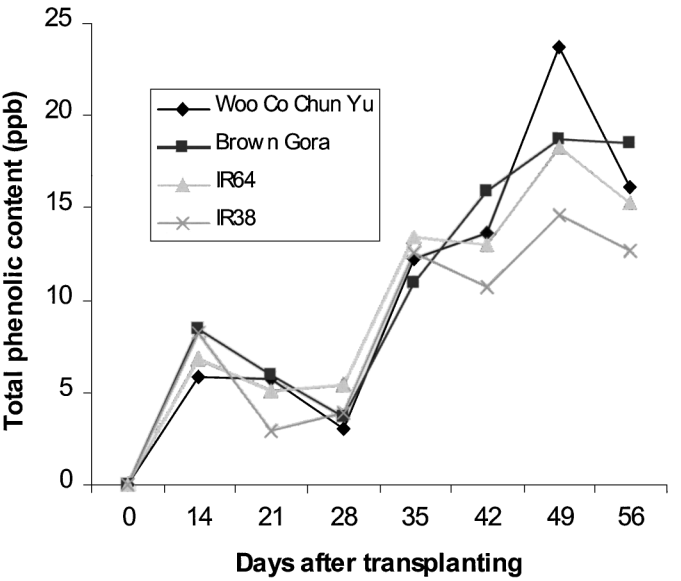


FIG. 2. Release of phenolic compounds (μg/plant/day) in traditional (Woo Co Chun Yu and Brown Gora) versus modern (IR64 and IR38) and allelopathic (Woo Co Chun Yu and IR64) versus nonallelopathic (Brown Gora and IR38) rice cultivars into hydroponic solution.

(Tanaka et al., 1990). As stated by Tsutsuki (1984), phenolic acids are phytotoxic at lower concentrations than are aliphatic organic acids, which are more common in decomposing rice straw than are phenolic acids and have often been evaluated for their autophytotoxic effects on rice (Gotoh and Onikura, 1971; Niranjan Rao and Mikkelsen, 1976). Our results indicate that it is unlikely that allelopathic interaction in rice is the effect of released phenolic acids alone, as the concentration needed for phytotoxic effects far exceeds the concentrations found in natural rice soils. Likewise, Gotoh and Onikura (1971) and Niranjan Rao and Mikkelsen (1976) found that released concentrations of aliphatic acids were too low to cause rice autotoxicity. However, rice autotoxicity (decomposing residues) may well involve wholly different processes than does rice allelopathy towards weeds (active root secretion).

The capacity of *p*-hydroxybenzoic acid to inhibit weed growth at concentrations that do not inhibit rice growth suggests that there has been an evolutionary drive in rice towards tolerance of phenolic acids. This could indicate that rice is better adapted than *Echinochloa* to grow under conditions of elevated phenolic acid concentrations, for example, in submerged (or lowland) rice soils during early growth stages. Such a selective response to phytotoxins is widely seen with herbicides (Streibig, 1988) and is often attributed to reduced uptake of the phytotoxin

(*p*-hydroxybenzoic acid in this case) or a more efficient metabolic degradation. However, the fact that the concentrations needed to inhibit *Echinochloa* growth are much higher than those exuded by rice cultivars again points towards a limited involvement of phenolic acids in allelopathic weed suppression under field conditions (Olofsdotter et al., 1999). Consequently the tolerance of rice cultivars to high phenolic acid concentrations is more likely to be an evolutionary response to phenolic acids formed during decomposition of plant residues than to phenolic acids exuded as allelochemicals.

It has been debated whether allelopathy under field conditions results from a single compound or a mixture of several phytotoxins (Einhellig, 1999). If a mixture of compounds is responsible for the observed growth inhibition of weeds, phenolic acids such as *p*-hydroxybenzoic acid could play a role in such a mixture. To fully establish the potential phytotoxicity of such mixtures, however, it is important to understand the joint action of these compounds. If allelopathy indeed results from mixtures of compounds, then evaluation of one phenolic acid at a time, as done here, cannot lead to proper identification of the causal agents or to determination of critical concentrations of all involved chemicals in field settings. Simultaneously, the results of this study will serve as a useful foundation for future studies of phenolic acid mixtures.

Nevertheless, our results raise some uncertainty regarding the role of phenolic acids in general and *p*-hydroxybenzoic acid in particular as a primary allelopathic agent of lowland rice against weeds. First, as described above, our measured rates at which phenolic acids are released from rice would require unrealistic quantities of time and plants in order to reach concentrations phytotoxic for weeds as determined in the dose–response curves. Second, the rate of phenol release does not differ between allelopathic and nonallelopathic rice cultivars. Third, dose–response curves do not indicate that traditional rice varieties are better adapted to phenolic acids than are improved varieties or that lowland rice varieties are better adapted than upland varieties. Fourth, the concentrations of phenolic acids released from all varieties into hydroponic solutions do not appear to notably exceed those resulting from straw decomposition (Chou et al., 1977; Tanaka, 1990), although differences in experimental design introduce some uncertainty in comparing data across studies. Thus, many weeds adapted to rice soils should tolerate the levels of exuded phenolic acids reported here, at least for short periods of time.

In this study, an admittedly limited numbers of rice cultivars and weed species were studied. However, the cultivars were chosen from previous allelopathy screenings (Navarez and Olofsdotter, 1996) to represent a wide range of allelopathic strength and a large genetic background. For the weed species, we selected some of the most troublesome weeds in rice cropping systems adapted to anaerobic (*E. crus-galli*) and aerobic (*E. colona*) growth conditions. The fact that the two species are both *Echinochloa* should make this comparison of aerobic and anaerobic conditions more valid.

Some limitations exist in synthesizing our results with other allelopathy studies. The tolerance levels of rice cultivars to *p*-hydroxybenzoic acid and their rates of phenolic acid release determined here cannot be compared with many equivalent measurements reported elsewhere for field settings. The type of extractant used in field studies will considerably affect the quantity of phenolic acids extracted, and there is no widely accepted method for isolating labile phenolic acids (Dalton, 1999). Soil moisture level and various interactions between phenols and soil components will affect phenolic acid activity (Foy, 1999; Huang et al., 1999; Schmidt and Ley, 1999). Phenolic acids degrade quickly in the field, and they can be leached (Tsutsuki, 1984), reducing their effective concentrations below exuded concentrations measured in solution cultures as in this study, which again suggests that the measured release rates for phenolic acids in this study cannot explain the allelopathy seen in rice. The correlation between phenolic acid content of rice and allelopathic activity of the same cultivars reported by Mattice et al. (1998) must, therefore, be explained either by the incidental correlation between phenolic acid content of the limited number of cultivars included in their study with their field suppressive activity, or it can be speculated that there might be genetic links between allelopathy and the phenolic acid content in rice, which per se do not link phenolic acid phytotoxicity with allelopathy.

Significant knowledge gaps remain in our understanding of rice allelopathy against weeds. Future studies must address the identification of allelopathic chemical(s) in rice as well as problems in translating laboratory results into field processes. Identification of allelochemicals in rice must also discern the true causal agents of allelopathy from a multitude of other chemicals that might be exuded from allelopathic cultivars, preferably without prior assumptions about which chemicals are active (Rimando et al., 1998).

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CHANGES IN THE GLUCOSINOLATE–MYROSINASE DEFENSE SYSTEM IN *Brassica juncea* COTYLEDONS DURING SEEDLING DEVELOPMENT

S. K. WALLACE[1,2] and SANFORD D. EIGENBRODE[1,*]

[1]*Department of Plant, Soil, and Entomological Sciences*
University of Idaho
Moscow, Idaho 83844-2339

[2]*Present address: Department of Entomology*
Montana State University
Leon Johnson Hall, P.O. Box 173020
Bozeman, Montana 59717-3020

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Abstract—Optimal defense theory (ODT) predicts that plant defenses will be allocated to plant organs and tissues in proportion to their relative fitness values and susceptibilities to attack. This study was designed to test ODT predictions on the myrosinase–glucosinolate defense system in *Brassica juncea* by examining the relationships between the fitness value of *B. juncea* cotyledons and the levels and effectiveness of cotyledon defenses. Specifically, we estimated fitness value of cotyledons during plant development by measuring plant growth and seed production after cotyledon damage or removal at successive seedling ages. Cotyledon removal within five days of emergence had a significant impact on growth and seed production, but cotyledon removal at later stages did not. Consistent with ODT, glucosinolate and myrosinase levels in cotyledons also declined with seedling age, as did relative defenses against a generalist herbivore, *Spodoptera eridania*, as estimated by bioassay. Declines in glucosinolates were as predicted by a passive, allometric dilution model based on cotyledon expansion. Declines in myrosinase activity were significantly more gradual than predicted by allometric dilution, suggesting active retention of myrosinase activity as young cotyledons expand.

Key Words—*Brassica juncea*, cotyledon, glucosinolates, myrosinases, plant defense, plant fitness, secondary metabolites, optimal defense theory, *Spodoptera eridania*.

*To whom correspondence should be addressed. E-mail: sanforde@uidaho.edu

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INTRODUCTION

Optimal defense theory (ODT) predicts that defenses should be allocated to plant tissues in proportion to the value of these tissues for plant fitness and their vulnerability to herbivore or pathogen attack (McKey, 1974; Feeny, 1976; Rhoades and Cates, 1976; Rhoades, 1979, 1983). The theory also predicts that defense allocation should change dynamically as fitness value and risk of attack of plant tissues change during plant development. We tested ODT predictions for the glucosinolate–myrosinase defensive system in cotyledons of *Brassica juncea* L.

Glucosinolates are nonvolatile nitrogen and sulfur-containing secondary metabolites derived from amino acid precursors (Louda and Mole, 1991). Glucosinolates are antibacterial, fungistatic, allellopathic, and toxic to some insects and mammals; however, they are also feeding stimulants for specialist insects such as flea beetles (Louda and Mole, 1991).

Hydrolysis of glucosinolates to glucose, sulfate, and volatile mustard oil products is catalyzed by enzymes known as myrosinases (thioglucoside glucohydrolases, EC 3.2.3.1). Myrosinases appear in all glucosinolate-producing plants studied, with the highest activity in seeds and seedlings (Bones, 1990; Rask et al., 2000). Myrosinase is located in special myrosin cells located throughout the plant (Xue et al., 1995), and glucosinolates are present in most cells. Damage to the integrity of the plant cell walls unites glucosinolates and myrosinases, allowing release of hydrolysis products (Cole, 1975; Feeny, 1977; Bones and Rossiter, 1996). Glucosinolate hydrolysis products are broadly biocidal, much more so than the intact glucosinolates. They are toxic to generalist insect herbivores (Seo and Tang, 1982; McCloskey and Isman, 1993; Borek et al., 1995, 1997, 1998; Li et al., 2000) and to crucifer specialist insects (Åhman, 1986; Wadleigh and Yu, 1988; Li et al., 2000) and have been implicated in defense against microbial diseases (Brabban and Edwards, 1995). Thus, glucosinolates and the myrosinase enzymes that catalyze their hydrolysis products appear to be important for full expression of binary glucosinolate-based defenses.

A few studies have documented the ecological importance of myrosinases for glucosinolate-based defenses. Newman et al. (1992) showed that thermally denaturing myrosinases in watercress, *Nasturtium officinale*, reduced defenses in this plant against arthropod herbivores. Recently, Li et al. (2000) showed that varying myrosinase activity and glucosinolate concentrations in *B. juncea* cotyledons significantly affected the area damaged by generalist *Spodoptera eridania* (Cramer) (Noctuidae) and specialist *Plutella xylostella* (L.) (Plutellidae) and the feeding frequency of *P. xylostella*.

Glucosinolates are categorized as a relatively inexpensive qualitative defense (Feeny, 1976; Rhoades, 1983) because their concentration in tissue is relatively

low. Chew and Rodman (1979) estimated the cost of glucosinolate synthesis at approximately 90 ATP and 12 $NADH_2$, which accounts for an isoleucine precursor and nine subsequent enzymatic steps to the glucosinolate product. However, the costs of maintaining glucosinolates for defense include synthesis, storage, compartmentalization, and possibly transport (Chew and Rodman, 1979; Gershenson, 1994; Bones and Rossiter, 1996; Hartmann, 1996). In addition, glucosinolate defense depends upon myrosinases, which may be the more costly component of the system. As evidence for this, *Brassica rapa* populations selected for high myrosinase activity produce less seed than low-myrosinase-activity genotypes (Siemens and Mitchell-Olds, 1988).

The glucosinolate-myrosinase system, therefore, has apparent metabolic costs, contributes to defense, and thus should be shaped towards optimal allocation by these opposing selective forces. Cotyledon tissue is particularly suitable for examining the optimality of glucosinolate defense allocations during plant development. Among the highest within-plant glucosinolate concentrations and myrosinase activities occur in cotyledons of *B. juncea* and *B. napus*, respectively (Bones, 1990; Bodnaryk, 1992). High allocations of defensive compounds to cotyledons is predicted by ODT because cotyledons are valuable storage organs for the growing seedling, the first photosynthetic tissue, and the first above-ground tissue available for terrestrial herbivore or pathogen attack. Flea beetle (*Phyllotreta cruciferae*) attack of *Brassica napus* and *B. rapa* is most detrimental at the cotyledon and first true leaf stage (Lamb, 1989). As the plant develops true leaves, the importance of cotyledons for plant fitness should decline, and ODT predicts that allocation of defenses to cotyledon tissues should also decline.

Many cotyledon studies have been performed on economically important *Brassica* crops, including *B. juncea* (Bodnaryk and Palaniswamy, 1990; Bodnaryk, 1992; Brandt and Lamb, 1993), but there is little reported on the important binary glucosinolate/myrosinase defense.

To test whether within-plant defenses are allocated to tissues in proportion to the specific tissue value, we measured the changes in the fitness value of *B. juncea* cotyledons during seedling development as determined by the effects of damage or cotyledon removal on ultimate plant height and seed production (tissue value estimation reviewed in Chew and Rodman, 1979; McKey, 1979; Krischik and Denno, 1983; Zangerl and Bazzaz, 1992). We compared these estimates of the fitness value of cotyledons with quantitative measurements of myrosinase activity and glucosinolate concentration in cotyledons during seedling maturation. We also measured the defenses of the cotyledons functionally through growth and feeding bioassays with a generalist herbivore. We hypothesized that estimates of the fitness value of cotyledons would decline during seedling development and predicted a similar decline in the levels of chemical defense, measured directly and in terms of response by an herbivore.

METHODS AND MATERIALS

Plants. Three *B. juncea* homozygous lines (designated 24, 41, and 9) were used in this study to broaden the domain of inference. Each was developed from the F_1 of a cross between the East Indian cultivar RLM514 and a breeding line from the University of Alberta (Thiagarajah and Stringam, 1993). Seed of these lines was provided by G. Stringam, University of Alberta, Edmonton, Alberta, Canada. Glucosinolate concentrations and myrosinase activity in these lines and their parental lines had been previously determined (Li et al., 2000, 2001). Glucosinolate profiles are >90% allyl and <10% 3-butenyl.

Seeds were hydrated on moistened filter paper-lined Petri dishes two days before sowing. All plants were grown in 15-cm-diam. pots in Sunshine Mix 1 potting soil (SunGro Horticulture, Bellevue, Washington), in a regulated greenhouse at $25 \pm 3^{\circ}$C, ambient humidity, with a supplemental 18L:6D photoperiod under metal-halide lighting (600 μmol PAR/m^2). Plants were watered daily by drip irrigation. One hundred forty-four plants of each line were used for chemical and biological assays. A second experiment to assess the effect of cotyledon damage on seed production and biomass included 70 plants of each line.

Insects. The generalist herbivore *S. eridania*, the southern armyworm, was used for bioassays. Insects were from a laboratory colony at the University of Idaho. The colony was reared on southern armyworm diet (Southland Products Inc., Lake Village, Alaska) in a growth chamber at 25°C with an 18L:6D photoperiod (light intensity 17.0 μEinstein/m^2/sec PAR, 400–700 nm).

Effects of Cotyledon Removal and Damage on Plant Growth and Seed Production. The effects of cotyledon damage or excision on plant fitness potential were investigated by damaging or removing cotyledons from plants at different times after plant germination and measuring plant growth (height and biomass at maturity) and seed production. The damage treatments were mechanical damage to both cotyledons with pliers (producing striated compression injury over the whole cotyledon surface) and removal of two cotyledons. The damage treatments were not intended to mimic insect damage, but to assess the effects of cotyledon injury or removal on subsequent plant growth and reproduction. Each of the treatments was applied 1, 5, or 10 days after germinating plants emerged from the soil. Plant height and total seed production were recorded at maturity. The small percentage (<1%) of undeveloped seed collected from mature plants was not included in our analysis. There were 10 replications; each replication included one randomly chosen plant of each of the three homozygous lines subjected to each of the six treatment combinations (2 damage types $\times$ 3 days applied), and an untreated control of each line, for a total of 210 plants. The plants were arranged on the greenhouse bench in a randomized complete block design.

Height and seed count data were compared with analysis of variance (ANOVA) based on a randomized design, with *B. juncea* line and treatment as main effects.

The analysis was conducted using PROC GLM and means were separated using the LSMEANS option (SAS Institute, Cary, North Carolina).

Myrosinase Activity and Glucosinolate Concentrations. Myrosinase activity was measured as in Li et al. (2000). Briefly, 20 half-cotyledons of each line were pooled and ground in 10 ml of pH 7.5 Tris EDTA buffer. Soluble proteins free of endogenous glucosinolates and sugars were obtained by filtration through a Sephadex G25 column. Exogenous sinigrin was added and glucose released by hydrolysis was measured colorimetrically using a glucose oxidase, peroxidase, 4-aminoantipyrine, and phenol reagent. Groups of extract samples were measured simultaneously in a microplate reader. Each sample was replicated twice in the plate. Absorbance at 490 nm was calibrated with a glucose standard curve included in each plate, and the signal for each extract sample was corrected for background absorbance with an extract without sinigrin control.

Glucosinolate concentrations were determined as in Li et al. (2000). Briefly, cotyledon halves were extracted in boiling methanol with PbBa acetate to precipitate phenolics and sulfate. Glucosinolates were then enzymatically desulfated and silylated with bis(trimethylsilyl)trifluoroacetamide (Sigma, St. Louis, Missouri). Trimethylsilyl derivatives were analyzed by gas chromatography with mass spectrometer detection using trimethylsilyl benzyl glucosinolate as an internal standard. Glucosinolates were identified by GC-MS (Hewlett Packard, HP 5890A gas chromatograph, coupled with a mass selective detector MSD 5972A; Agilent Technologies, Palo Alto, California) and a DB-5 capillary column (30 μm $\times$ 0.25 mm ID, 0.25-μm film thickness; J&W Scientific, Folsom, California). Glucosinolate compounds were identified based on standard mass spectra (provided by M. Morra, University of Idaho) and their relative retention times. Glucosinolate concentrations were quantified by GC with a flame ionization detector (HP 5890A), using a HP-5MS (30 μm $\times$ 0.25 mm ID, 0.25-μm film thickness; Agilent Technologies) or a SPB-5 (30 μm $\times$ 0.25 mm ID, 0.10-μm film thickness; Supelco, Bellefonte, Pennsylvania) capillary column.

Myrosinase activity and glucosinolate concentration data were analyzed similarly. Values from pooled samples of each line were used as a single observation, providing three observations for each estimate, except for day 5 (five days after emergence), which excluded line 9. ANOVA was performed on these data using a randomized design with cotyledon age as the class variable. Because secondary chemistry might change passively as the cotyledon expands, we also calculated an expected concentration based on simple dilution. This expected value was calculated by dividing the average mass of cotyledons on plants one day after emergence by the average mass of cotyledons at 5 or 10 days after emergence, respectively. These factors were then multiplied by the average glucosinolate concentration or myrosinase activity/unit mass on day 1 to estimate expected values at 5 or 10 days after emergence. Student's *t* test was used to examine whether

glucosinolate concentration or myrosinase activity 5 or 10 days after emergence differed significantly from this expected value.

Bioassay to Measure Cotyledon Defenses. Neonate larvae of *Spodoptera eridania* were placed five per cage in 2-oz clear plastic rearing cups with a layer of 6% water agar. The larvae were provided cotyledons from seedlings 1, 5, or 10 days after emergence daily from one of each *B. juncea* line. Despite differences in the size of cotyledons of different ages, the larvae never consumed an entire cotyledon before fresh tissue was provided. Old tissue and dead larvae were removed daily and recorded. After four days, surviving larvae were starved for one day, with access to water, and then weighed. There were 10 replications, totaling 50 caterpillars per line.

Caterpillar weights and survival at the end of the bioassay were analyzed with a two-factor ANOVA based on a completely randomized design with *B. juncea* line and cotyledon age as factors. Observations used in the analysis were the average weight of all surviving caterpillars in each rearing cup and the proportion of caterpillars surviving in each cup. No data were obtained for cotyledons from plants five days after emergence for *B. juncea* line 9. Because the effect of line and the line $\times$ day interaction were not significant, we also conducted single factor ANOVA for the effect of cotyledon age on weight and on survival, and separated the means with Tukey's HSD test at the 0.05 level. All statistical analyses were performed using PROC GLM and PROC MEANS in SAS (SAS Institute, Cary, North Carolina).

RESULTS

Effects of Cotyledon Removal and Damage on Plant Growth and Seed Production. Removal of cotyledon tissue from *B. juncea* seedlings affected both height at maturity and seed production (Table 1, Figure 1). Plants with removed cotyledons were shorter relative to controls (Figure 1A) and plant height was affected by treatment, line, and their interaction (Table 1). The effect of treatment was largely due to the reduced height of plants from which two cotyledons had been removed on either day 1 or day 5 (Figure 1A). The line effect and interaction were caused by a relative tolerance of line 23 (removal of two cotyledons from this line on day 1 did not significantly reduce height at maturity relative to undamaged controls) ($P = 0.066$, data not shown). Removing both cotyledons on day 5 only significantly reduced height of line 41 ($P = 0.003$) and not the other two lines (data not shown).

Plant seed production was affected by treatment and line but not by their interaction (Table 1). As was true for plant height at maturity, removal of cotyledons within five days of emergence substantially reduced seed production (Figure 1B).

TABLE 1. ANOVA RESULTS FOR EFFECTS OF COTYLEDON DAMAGE OR REMOVAL ON PLANT HEIGHT AND SEED SET IN THREE LINES OF *Brassica juncea*

Source	*df*	*MS*	*F*	*P*
Response: plant height at maturity				
Model	29	6346.889	6.00	0.001
Error	181	1057.442		
Block	9	6015.661	5.69	0.001
Line	2	5208.189	4.93	0.008
Treatment	6	15307.3063	14.48	0.001
Line * TRT	12	2360.443	2.23	0.012
Response: seed production				
Model	29	478288.9	4.51	0.001
Error	181	106023.9		
Block	9	542887.3	5.12	0.001
Line	2	437769.5	4.13	0.017
Treatment	6	1175429.6	11.09	0.001
Line * TRT	12	95375.2	0.90	0.549

In summary, plant height at maturity and seed production were reduced by cotyledon removal from seedlings one or five days after emergence. Cotyledon damage at any stage or cotyledon removal after day 5 did not significantly reduce growth or eventual seed production of *B. juncea* seedlings.

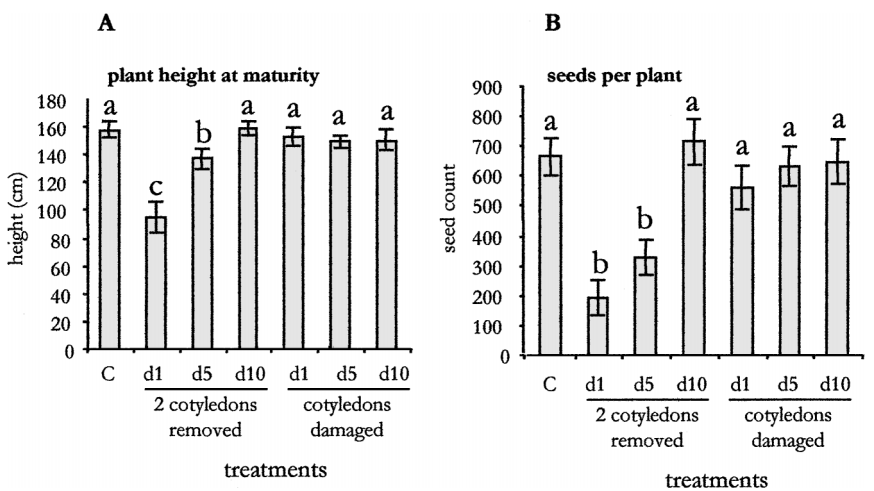


FIG. 1. Effects of cotyledon removal and damage at different seedling ages on *B. juncea* plant height at maturity (A), and per-plant seed production (B). In each panel, values with the same letter are not significantly different ($P \leq 0.05$); bars are SEM.

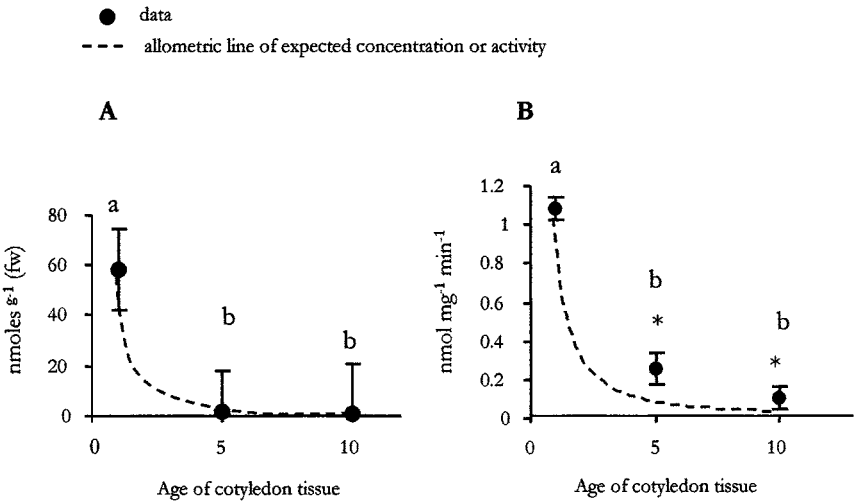


FIG. 2. Glucosinolate activities (A) and myrosinase concentrations (B) in cotyledons of *B. juncea* seedlings 1, 5, and 10 days after emergence. The data are for three *B. juncea* lines pooled. The allometric trendline shows expected specific activity of myrosinase or expected concentrations of allyl glucosinolate based on dilution due to cotyledon expansion (see text). In each panel, values with the same letter are not significantly different ($P \leq 0.05$). Asterisks show significant differences between observed values and those expected due to dilution ($P \leq 0.05$); bars are SEM.

Glucosinolate concentrations in cotyledons also decreased with seedling age (*df* 2,7; $F = 31.36$; $P = 0.001$) between day 1 and day 5 (Figure 2A). The falling concentrations were not different from those predicted from simple allometric dilution, suggesting that the growth of the cotyledon tissue rather than catabolism or active transport of glucosinolates served to alter defense concentrations.

Defensive Chemistry: Glucosinolate Concentrations and Myrosinase Activity. Myrosinase activity, in nanomoles of glucose released per microgram of fresh tissue per minute, was affected by the age of cotyledons (*df* 2,5; $F = 59.78$; $P = 0.0003$) (Figure 2B), decreasing with seedling age. Myrosinase activity was significantly higher than predicted from simple allometric dilution of myrosinase activity as cotyledons expanded ($P < 0.001$).

Bioassay to Measure Cotyledon Defenses. Weights of larvae fed cotyledons aged one day were lower than those aged 5 or 10 days (Figure 3A). Survivorship of *S. eridania* was lower when fed cotyledon tissue from 1-day-old seedlings than when fed cotyledons from 5 or 10-day-old seedlings (Table 2) (Figure 3B). Based on survival and growth rates of the generalist *S. eridania*, defenses in *B. juncea* cotyledons were highest in young cotyledons and declined as seedlings matured through 10 days (Figure 3). The caterpillars that survived on the

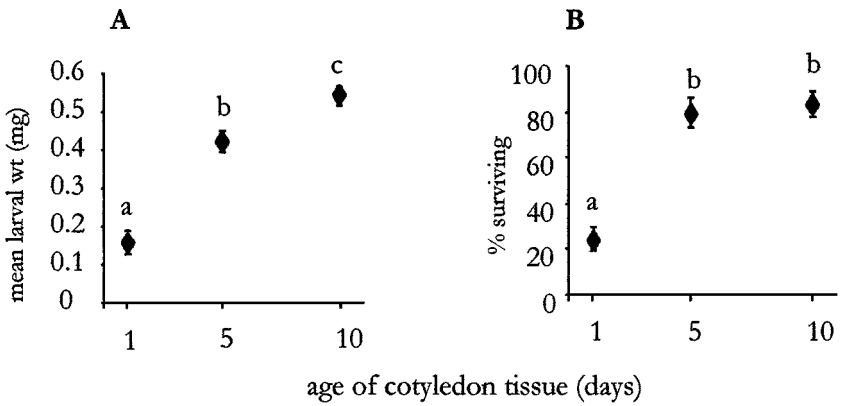


FIG. 3. Larval weights (A) and survival (B) of *S. eridania* fed *B. juncea* cotyledons from seedlings 1, 5, and 10 days after emergence. In each panel, values with the same letter are not significantly different ($P \leq 0.05$), bars are SEM.

treatments appeared not to eat or to eat little of the cotyledon tissues from 1-day-old plants and therefore did not gain weight. It could not be determined if reduced feeding was due to feeding deterrence or postingestive effects of the cotyledon tissues.

DISCUSSION

Attempts to confirm aspects of optimal defense theory have generated sizable literature (Simms, 1992; Zangerl and Bazzaz, 1992; Sagers and Coley, 1995; Zangerl and Rutledge 1996; Ohnmeiss et al., 2000) and include studies on glucosinolate-producing plants and plant parts during different growth stages

TABLE 2. ANOVA RESULTS FOR EFFECTS OF COTYLEDON AGE ON SURVIVAL AND GROWTH OF *Spodoptera eridania* NEONATE LARVAE

Source	*df*	*MS*	*F*	*P*
Response: caterpillar weight				
Day	2	0.759	52.43	0.001
Line	2	0.001	0.06	0.938
Line * Day	3	0.039	2.7	0.054
Residual	56	0.014		
Response: percent survival (arcsine transformed)				
Day	2	5.931	30.15	0.001
Line	2	0.266	1.35	0.266
Line * Day	3	0.426	2.16	0.100
Residual	72	0.197		

(Bodnaryk, 1992; Lamb et al., 1993; Palaniswamy and Lamb, 1993), as well as studies on myrosinase activity in different plant parts during development (Bones, 1990; Lenman et al., 1990; James and Rossiter, 1991; Falk et al., 1992; Xue et al., 1995). Our study examines three elements of ODT for plant tissues defended by the glucosinolate–myrosinase defensive system. We measured both glucosinolates and myrosinase simultaneously during cotyledon development of *B. juncea* and the actual level of defense of these tissues against herbivory using a bioassay with a generalist herbivore, and we estimated the fitness value of the cotyledon tissues in terms of plant growth and seed production.

Our data show allocation of the binary glucosinolate–myrosinase defense to cotyledon tissues is broadly consistent with the ODT prediction that within a plant the defenses are allocated to tissues in proportion to the tissue value. High initial myrosinase activity and glucosinolate concentrations in cotyledons occurred when these tissues were most critical for plant fitness, as estimated by the effects of removing cotyledons on plant height at maturity and seed production. As the value of cotyledons for plant performance (growth and seed production) declined, both components of this binary defense system also declined.

Consistent with these dynamics in defensive chemistry, *S. eridania* growth and survival were lowest when fed young cotyledons and increased with increasing age of the cotyledons eaten. Although the defensive characteristics responsible for this pattern cannot be determined from our experiment, it has been shown that allyl glucosinolate, the dominant one in the three *B. juncea* lines tested, is toxic to *S. eridania* neonate larvae ($LC_{50} = 6.73$ μmol/g diet) and that the myrosinase catalyzed hydrolysis product, allyl isothiocyanate has a higher toxicity ($LC_{50} = 3.42$ μmol/g diet) (Li et al., 2000). This suggests that the glucosinolate–myrosinase system contributes to *B. juncea* defense against *S. eridania* and that the decline of the system components in cotyledons as seedlings mature results in the observed declining defense of these tissues.

The dynamics of glucosinolate concentration in *B. juncea* cotyledons can be explained by a passive dilution with the expansion of young cotyledon tissues (our data, Bodnaryk and Palaniswamy, 1990). Thus, initial seed defense allocations apparently serve the tissue throughout its growth (Figure 3). This contrasts with conclusions of a similar study of glucosinolate dynamics in seedling *Brassica napus*, which also displays a rapid decline in seed glucosinolate concentrations in the first seven days of growth (Clossais-Besnard and Larher, 1991). These authors postulate that seedling glucosinolates are rapidly metabolized for subsequent plant growth, constituting a sink for carbon, nitrogen, and sulfur, serving two or more functions within the plant, aside from defense (see also Bones and Rossiter, 1996). The data presented by Clossais-Besnard and Larher (1991) do not permit examination for a possible passive dilution effect for *B. napus* seedlings.

Myrosinase activity levels did not decline as predicted by a passive allometric model, but remained somewhat elevated over expected levels, at a presumed

cost to plant fitness. Myrosinases occur in complexes and function using accessory components such as myrosinase-binding proteins and epithiospecifier proteins (Bones, 1990; Bones and Rossiter, 1996; Taipalensuu, 1996). During hydrolysis, an epithiospecifier protein (ESP) interacts with myrosinase to specify the reaction products of the glucosinolate–myrosinase interaction (Bones, 1990). *Brassica juncea* was found to have ESP activity (Cole, 1975). These accessory components must be accounted for in a quantitative analysis of the cost of defense. The elevation of myrosinase activity above allometric expectations could reflect changes in some of these associated factors, or through synthesis of additional myrosinase enzymes.

However they arise, levels of myrosinase activity above those expected from passive dilution may be adaptive for defense. 3-Indolylmethyl- and 4-hydroxy-3-indolylmethylglucosinolates can be induced approximately 3-fold and 1.5-fold, respectively, in *B. juncea* cotyledons (Bodnaryk, 1992). Myrosinase activity in *B. juncea* cotyledons is not induced by herbivory or mechanical damage (Byrne and Eigenbrode, unpublished data). Thus, the elevated levels of myrosinase activity in older cotyledons may complement these inducible glucosinolates in the event of pathogen or herbivore attack.

Our study on the dynamics of myrosinase and glucosinolates in *B. juncea* cotyledon tissue must be interpreted realizing that the test lines were developed from agronomic cultivars (Thiagarajah and Stringam, 1993) and that the greenhouse environment used for the test lacks some of the stresses likely to be important for plant growth and seed production in the field. Furthermore, our damage treatments do not model many types of herbivory, and feeding by different insects differs in its impact on plant fitness. Feeding by seven different insect species differed in its impact on seedlings of canola, *Brassica napus*, and mustard, *Sinapis alba* (Gavloski and Lamb, 2000). Nonetheless, tested patterns of defense allocation we have measured in *B. juncea* cotyledons appear qualitatively optimal.

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DEPLETION OF HOST-DERIVED CYANIDE IN THE GUT OF THE EASTERN TENT CATERPILLAR, *Malacosoma americanum*

T. D. FITZGERALD,[1,]* P. M. JEFFERS,[2] and D. MANTELLA[2]

[1]*Department of Biological Sciences*
[2]*Department of Chemistry*
State University of New York at Cortland
Cortland, New York 13045

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Abstract—Using a colorimetric procedure, we assessed the HCN-p of black cherry leaves (*Prunus serotina*) ingested by the eastern tent caterpillar, *Malacosoma americanum*, and the cyanide content of the bolus as it passed thorough the caterpillar's digestive tract and into the detritus pool. The mean HCN-p of leaves in our study area was 1902 ± 174 (SE) ppm. Young leaves found at the tips of growing branches, which the caterpillars preferred, had a significantly higher HCN-p (3032 ± 258 ppm) than older leaves found at the middle (1542 ± 243 ppm) or base of the shoot (1131 ± 159 ppm). Following a bout of overnight feeding on young leaves, the cyanide content of the foregut and midgut boluses of early sixth-instar caterpillars averaged 631 ± 161 ppm, and 14 ± 3 ppm, respectively, indicating that host-derived cyanide is rapidly depleted as the bolus transits the gut. Some cyanide, however, remains. In three studies, the mean cyanide content of fresh fecal pellets ranged from approximately 20 to 38 ppm, while the dried, compacted pellets ranged from 63 to 85 ppm. Food in the foreguts of mature caterpillars dispersing over the ground in search of pupation sites had 417 ± 99 ppm cyanide. The potential impact of this egested and caterpillar-transported cyanide on the consumer and detritivore communities is discussed.

Key Words—*Malacosoma americanum*, tent caterpillar, cyanide, *Prunus serotina*, black cherry, Lasiocampidae.

*To whom correspondence should be addressed.

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INTRODUCTION

During the spring of 2001, horse breeders in central Kentucky experienced an unprecedented loss of the current year's foals, a phenomenon that became know as mare reproductive loss syndrome (MRLS) (Kane and Kirby, 2001). After ruling out the involvement of infectious agents, a toxin was implicated and the investigators eventually focused on the possibility that the loss of foals was due to cyanide poisoning. The likely source of the cyanide was the black cherry tree *Prunus serotina*. Concurrent with the epidemic was a local population explosion of tent caterpillars, *Malacosoma americanum*, leading some to believe that the caterpillar was also involved in the problem (Kane and Kirby, 2001). Although reports of a link between the direct consumption of cherry leaves and the mortality of livestock have appeared in the scientific literature for over 100 years (e.g., Morse and Howard, 1898; Smeathers et al., 1973), there have been no studies implicating tent caterpillars in the process. Indeed, there have been no reports of any vertebrate suffering from cyanide toxicity after ingesting tent caterpillars or their fecal pellets. Nonetheless, *P. serotina*, a particularly cyanogenic species of cherry, is a favored host of *M. americanum* (Fitzgerald, 1995), and Peterson et al. (1987) showed that the defensive regurgitation of the newly fed caterpillar contained HCN derived from the cyanogenic precursor prunasin, which occurs in the leaves of the tree. When a leaf is mechanically damaged by a caterpillar, prunasin is converted to the cyanohydrin, mandelonitrile, which subsequently disassociates to form benzaldehyde and the toxin. The caterpillar itself is immune to ingested or inhaled cyanide and is presumed to detoxify it (Fitzgerald, 1995), but there have been no attempts to measure the amount of cyanide in the bolus as it passes through the digestive tract of the insect. Such information is particularly relevant to the possible involvement of caterpillar-transported or egested cyanide in MRLS, but it also has larger ecological implications because tent caterpillars may fall prey to small vertebrate predators, and the fecal pellets of the insects then enter the detritivore and decomposer food chains. We undertook the present study to determine the HCN-p of leaves of black cherry ingested by the eastern tent caterpillar, *Malacosoma americanum*, and the cyanide content of the bolus as it passed thorough the caterpillar's digestive tract and into the detritus pool.

METHODS AND MATERIALS

Study Site. The study was conducted during the spring and summer of 2001. Colonies of *M. americanum* and leaves of *P. serotina* used in the study were collected from various field locations in Cortland County, New York, as described below.

Chemical Analysis of Cyanide. Studies we conducted preliminary to our chemical assays indicated that the small samples we used (typically 3–6 mg)

could be efficiently and rapidly homogenized with a micro tissue-grinder (barrel length, 5 cm, barrel diameter, 0.3 cm) with no significant loss of cyanide. Although a previous study (Torres, et al., 1988) indicated that the most efficient extraction of cyanide from plant tissue occurs when the material is first ground to a powder in liquid nitrogen, test samples of leaves we processed with the micro tissue-grinder in our preliminary study had cyanide yields that were not significantly different from those we obtained by grinding the tissue in liquid nitrogen (paired t test, $T = -1.60$, $P = 0.15$, $N = 8$ pairs of leaf samples).

To prepare our samples for distillation, the material was placed in the bottom of the grinder and ground with 0.5 ml of 0.1 M phosphate buffer ($NaH_2PO_4 \cdot H_2O$, pH 6.8) (Brinker and Seigler, 1989, 1992) and chilled to approximately 4°C in an ice bath. The grinder was kept chilled up to the moment of grinding by parking it in another ice bath. The sample was ground until fully homogenized, a process requiring 5–10 sec depending on the nature of the material. The buffer was then transferred to the outer compartment of a Warburg flask (capacity = 18 ml), the center compartment of which was preloaded with a measured quantity of 1 M NaOH. The micro tissue-grinder was immediately rinsed with another 0.5 ml of chilled buffer, and the rinse was added to the flask. The flask was sealed and held in a temperature-controlled cabinet at 35°C for a minimum of 18 hr. During the distillation process, HCN volatilizing from the sample was trapped in the NaOH as nonvolatile CN^-. More detailed procedures for specific sample types (leaves, fecal pellets, and gut contents) are given below.

We used the colorimetric technique of Lambert et al. (1975) as modified by Brinker and Seigler (1989, 1992) to determine the quantity of cyanide in our samples. Briefly, an aliquot of the 1 M NaOH solution was transferred from the center well of the Warburg flask to a test tube, and the solution was adjusted to 0.1 M NaOH. Measured quantities of 1 M acetic acid sufficient to neutralize the NaOH, a succinimide–*N*-chlorosuccinimide oxidizing reagent, and a barbituric acid–pyridine coupling reagent were then added to the tube in succession. Following vortexing, the solution was allowed to develop for 10 min, and then the absorbance was read at 580 nm on a Spectronic 20 spectrophotometer. Standard curves relating absorbance to cyanide quantity were prepared following the procedures of Brinker and Seigler (1989).

Preparation of Samples. The HCN-p of variously aged leaves of *P. serotina* was determined. Vigorously growing branches, all bearing young expanding leaves at their apices were collected from three to five trees from four distinct field populations located in Cortland County, New York. Whole branches were cut from the trees, and the cut ends of the stems placed in containers of water to prevent wilting. Care was taken to collect no more branches at one time than could be processed within 1 hr of collecting to minimize the possibility that cutting the branch would induce the production of cyanide. A 3- to 6-mg section was cut from the blade of the youngest, partially expanded leaf located at the tip of the branch,

another from a leaf at the middle of the branch, and another from the oldest leaf found at the base of the branch. Sections were separately ground and sealed in Warburg flasks as described above.

It is not known if damage to cherry trees can induce the production of cyanide, as reported for other allelochemicals from other tree species. Since cutting branches necessarily involves some systemic disturbance, we conducted a preliminary study to determine the likelihood that our procedure induced cyanide production in our sample leaves. A young leaf from near the apex of a shoot was selected at random from a tree growing near our laboratory and cut in half with scissors. The cut half was taken immediately to the laboratory and a section excised, weighed, ground, and sealed in a Warburg apparatus as described above. Twenty-four hours later the half remaining on the tree was collected and treated in the same manner as the first half. To determine if this procedure induced cyanide production, we compared the cyanide content of the two halves. The experiment was replicated five times with leaves from different trees.

The cyanide content of food in the foreguts and midguts of fifth-instar caterpillars allowed to feed ad libitum overnight in the laboratory was determined. Caterpillars were maintained in colonies on wooden frames (Fitzgerald, 1995) and provided daily with freshly collected branches of *P. serotina*. The caterpillars of *M. americanum* are finicky feeders (Fitzgerald 1995), and care was taken to provide colonies with branches bearing the younger leaves the caterpillars prefer. Colonies were maintained at $21 \pm 2^{\circ}$C under a 10L:14D daylight fluorescent light regime with lights on at 08:00 hr. Caterpillars were collected from the colonies at 09:00 hr and immediately frozen. Samples of the bolus of the foregut and midgut were obtained for chemical assay by cross sectioning with a razor blade whole frozen caterpillars in the mid-thoracic and mid-abdominal regions. The bolus in the exposed gut was removed with a forceps. Dissection was carried out on a cold-table at approximately -10°C, allowing the bolus to be removed, weighed, and placed in the micro tissue-grinder while still frozen.

The cyanide content of the foregut bolus was measured at two different times to determine if loss of HCN occurs in this section of the digestive tract. Forty caterpillars maintained in a colony and allowed to feed ad libitum overnight, as described above, were collected at 09:00 hr. Half of the caterpillars were immediately frozen, the other half were placed individually in Petri plates and held without food until 16:00 hr, then frozen. The foregut was subsequently removed by allowing the cadaver to thaw to just above freezing, grasping it by the head, and pulling the head off and with it the attached foregut. This technique was used in preference to dissection because it produced a clean gut: the dense fat body that surrounds the gut of the sixth instar was left behind in the hemocoel. Once exposed, the gut was laid out on the cold table, cut at its junction with the midgut, a sample taken, weighed, ground, and sealed in a Warburg flask for distillation.

Full-grown sixth-instar caterpillars dispersing over the ground were collected over a nine-day period in early June from roadways at two different locations in Cortland County. Dispersal occurs when caterpillar are nearing pupation and in search of pupation sites (Fitzgerald, 1995). The caterpillars were frozen within 15–30 min of collection. The foregut was subsequently removed, weighed, and a sample taken for analysis as described in the preceding paragraph.

The cyanide content of fresh and dried fecal pellets was determined in three separate studies. For the first study, 15 fifth-instar caterpillars were housed overnight in Petri plates with fresh cherry leaves. In the morning, all food and fecal pellets were removed from the dishes and discarded. Fecal pellets produced by each of the 15 caterpillars over the next 2 hr were collected and frozen for later analysis. A separate group of 15 caterpillars was treated similarly, but the fecal pellets were allowed to dry for 4–6 hr after deposition before they were collected and frozen. Frozen pellets were subsequently placed directly in the micro tissue-grinder, ground, and their cyanide content collected by distillation in the Warburg apparatus. Because the cyanide content of the fecal pellet is small, sets of two pellets were used for each of the 15 replicates of each treatment.

In the above study, the fresh and dried fecal pellets were not weight-matched, and the data collected from the study could not be used to determine if the fecal pellets lost cyanide as they dried. Another study was conducted to examine this question. Following a bout of feeding, several hundred fresh fecal pellets produced by a whole colony of fifth-instar caterpillars were collected within 2 hr of egestion. A sample of 15 of these pellets was weighed, then allowed to dry at 30°C in the open for several hours. The pellets were reweighed after drying and the ratio of fresh to dry weight calculated. A second sample of weight-matched pellets from this same source was used to calculate the cyanide content of fresh and dry pellets. Fifteen sets consisting of two fresh pellets and 15 sets consisting of two dry pellets were assayed. ANOVA showed no significant difference between the initial mass of the fresh pellets in the two sets ($F = 0.0015$, $P = 0.96$).

In a third study, the cyanide contents of fecal pellets extracted directly from the rectums of frozen sixth-instar caterpillars collected from roadways while dispersing were determined as described above.

Although it would appear likely that all of the cyanide recovered from caterpillars and fecal pellets was produced from cyanogenic precursors found in the leaves of *P. serotina*, we set up a control study in which we assayed for cyanide in the foregut bolus and in fecal pellets of caterpillars reared on an acyanogenic variety of apple (*Malus*), which is a natural alternate host of the caterpillar (Fitzgerald, 1995). We used the same analytical procedures as outlined above for caterpillars fed the leaves of *P. serotina*.

Bioassay of Cyanide Toxicity. To assess the residual toxicity of host material processed by the caterpillar, we compared the knockdown power of HCN

emanating from macerated cherry leaves with that of HCN emanating from macerated fecal pellets. A gas chamber was prepared by gluing together the bottoms of two 50-mm × 9-mm tight-lid plastic Petri plates, then drilling a series of holes through the connected bottoms. Ten second- to third-instar nymphs of the milkweed bug *Oncopletus fasciatus* were placed in the upper section of the chamber and the chamber sealed with a top. Approximately 0.5 g of dried fecal pellets collected from under a colony of fourth-instar tent caterpillars fed young cherry leaves in the laboratory were ground with a mortar and pestle in 2 ml of the phosphate buffer. The slurry was poured into the top of a second Petri plate, spread into a thin layer, and the top used to seal the bottom section of the chamber. Thus, HCN emanating from the slurry in the bottom section of the chamber entered the section containing the bugs by passing through the connecting holes. The 10 bugs were observed continuously for the next 2 hr, and the time when individuals first collapsed was recorded. This procedure was repeated with a slurry prepared by grinding approximately 0.5 g of partially expanded terminal leaves and another slurry made from a basal leaf of approximately the same mass. We also observed the response of bugs sealed in a chamber changed with the phosphate buffer alone.

Statistics. All statistical tests were performed with Prostat or Sigmastat software.

RESULTS

The cyanide content of samples of 48 leaves of *P. serotina* collected from 16 trees is shown in Table 1. The mean cyanide content for all leaf samples collectively was 1902 ± 174 ppm. There was no significant difference in the cyanide content of the two halves of cut leaves removed from the tree at different times, indicating that the relatively minor disturbances to the leaves associated with collecting our samples for assay was unlikely to have had a significant influence on the cyanogenic potential of the leaves (paired *t* test, $T = 1.78$, $P = 0.15$). Although we found no

TABLE 1. MASS AND CYANIDE CONTENT OF *Prunus serotina* LEAVES FROM 16 TREES[a]

Location on branch	Leaves (N)	Leaf mass (mg)	Cyanide (ppm)	Total cyanide content of leaves (mg)
Terminal	16	52.7 ± 5.5	3032 ± 258	0.16 ± .02
Medial	16	600.0 ± 85.2	1542 ± 243	0.89 ± 0.17
Basal	16	327.4 ± 54.4	1131 ± 159	0.40 ± 0.11

[a] Leaves were collected June 20–27, 2001, from four locations in central New York State. Values are mean ± SE.

evidence of cyanide induction in leaves that had been mechanically severed, it remains to be determined if caterpillar feeding induces cyanide production.

All 20 of the foreguts of caterpillars allowed to feed ad libitum overnight on young cherry leaves and killed at 09:00 hr contained food. The midguts of 16 of these also contained food, but four others were empty and were not used in the analysis. The 20 foregut boluses contained 631 ± 162 ppm of cyanide. The 16 midgut boluses contained 14 ± 3 ppm of cyanide. The difference is highly significant (ANOVA, $F = 10.82$, $P = 0.002$).

A comparison of the mass and cyanide content of the foreguts of two groups of 20 caterpillars allowed to feed ad libitum overnight and killed at either 09:00 hr or 16:00 hr showed that the foregut and its food load had a mass equivalent to $12.5 \pm 0.1\%$ of the total body mass of those killed at 09:00 hr and $5.2 \pm 0.1\%$ of those killed at 16:00 hr. The difference is significant (ANOVA of arcsin $\sqrt{P}$ transformations, $F = 22.52$, $P < 0.001$). All but three caterpillars killed at 09:00 hr had food in their foreguts. Ten of those with full guts were randomly selected for analysis. Nine killed at 16:00 hr had completely empty foreguts, while only eight had sufficient food in the foregut for analysis. The cyanide content of the foregut boluses from caterpillars killed at 09:00 hr was 1059 ± 125 ppm and for those killed at 16:00 hr, 175 ± 46 ppm. The difference is significant, indicating that the depletion of cyanide occurs in the foregut (ANOVA, $F = 36.19$, $P < 0.001$). The presence of food in the foregut of some caterpillars that had not fed for at least 7 hr is consistent with a previous study showing that 6 hr after feeding, residual food remained in the foreguts of eastern tent caterpillars maintained at 23°C (Casey et al., 1988). The rate of food processing in tent caterpillars is temperature dependent, occurring most rapidly in larvae allowed to raise their body temperature by basking and not at all in caterpillars whose body temperature is less than 15°C.

Of the dispersing sixth-instar caterpillars collected from roadways, 5 were males and 25 females. The total body mass of the males was 0.67 ± 0.03 g and the females, 1.13 ± 0.03. Nine of the 30 caterpillars had empty foreguts. For the remainder, the mean mass of the foregut was 0.09 ± 0.01 g. On a percentage basis, the foregut of these 21 caterpillars constituted $11.1 \pm 0.1\%$ of their total body mass. The foregut boluses contained 417 ± 99 ppm of cyanide.

The fresh fecal pellets of one group of 15 caterpillars contained 37 ± 4 ppm of cyanide. The dry fecal pellets of another group of 15 caterpillars contained 86 ± 15 ppm of cyanide. The ratio of fresh to dry weight for fecal pellets collected from a whole colony following a feeding bout was 3.4. Fresh fecal pellets from this colony contained 21 ± 1 ppm cyanide, while dry pellets contained 63 ± 7 ppm. Based on these values, the ratio of cyanide in fresh to dried pellets was 3.1. When the total amounts of cyanide in the dry and fresh pellets was compared, there was no difference (ANOVA, $F = 2.09$, $P = 0.16$). This indicates that there is no significant loss of cyanide from the pellet during the drying process. Although the pH of the gut of the tent caterpillar is unknown, the guts of lepidopterous

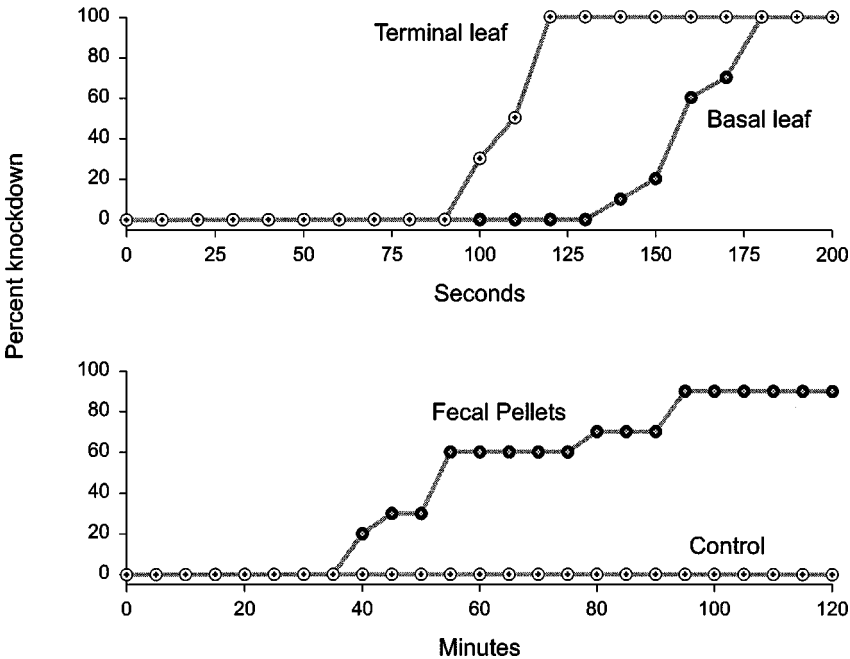


FIG. 1. Collapse of *Oncopeltus faciatus* nymphs in a gas chamber charged with slurries prepared from young or aged leaves of *Prunus serotina*, and from a slurry prepared from fecal pellets of *Malacosoma americanum* caterpillars reared on young leaves of *P. serotina*.

larvae typically have pH values of 8–12 (Chapman, 1998), and under such alkaline conditions, the HCN in the pellet or prepellet bolus should be converted to the relatively stable CN^-. It is also possible that pellets contain residual prunasin that is not converted to HCN until the pellet is homogenized. Fecal pellets taken directly from the rectum of 15 dispersing sixth-instar caterpillars had 19.8 ± 1.2 ppm cyanide.

The results of the bioassay with *Oncopeltus* is shown in Figure. 1. Consistent with our chemical analyses, knockdown took approximately 20–50 times longer when the bugs were exposed to a slurry of fecal pellets compared to slurries of leaves. Both the gut contents and the fecal pellets of tent caterpillars reared on *Malus* leaves were devoid of cyanide.

DISCUSSION

There have been several previous studies to determine the HCN-p of the leaves of *P. serotina*. Morse and Howard (1898) reported that in New Hampshire

the HCN-p of cherry leaves grouped into four classes, ranging from dry and woody to succulent, varied from 420 to 2120 ppm. Smeathers et al. (1973) indicated that the cyanide content of the leaves of *P. serotina* in Kentucky varied seasonally with an average 2472 ppm at the beginning of the season in April and 43 ppm at the end of the season in November, but that study provides no statistical measurement of variation. Peterson et al. (1987) sampled leaves on Long Island, New York. in May and June and found 1845 ± 154 (SE) ppm in young leaves and 1128 ± 202 ppm in mature leaves. Santamour (1998) reported that in Washington, D.C., from June to early September the HCN-p of *P. serotina* leaves was 2644 ± 236 (SE) ppm, but in November it declined to 209 ppm. He also reported that newly emerging leaves have an HCN-p of 7432, but gives no indication of sample size or variation. The broad variation in HCN-p found among and within trees in the present study (Table 1) is consistent with that reported in those studies. Aside from the singular measurement reported by Santamour (1998) for an emerging leaf, there have been no previous attempts to assess the HCN-p of the young tip leaves that eastern tent caterpillars prefer (Fitzgerald, 1995). Our study shows that these leaves are particularly cyanogenic. Under conditions of moderate infestation, these young leaves are likely to be completely harvested from a tree before the caterpillars turn their attention to the older leaves (Fitzgerald, 1995). During the first few larval stadia, the caterpillars are likely to subsist almost completely on these partially expanded leaves.

Our study shows that the amount of cyanide in the foregut bolus decreases over time and that food in the midgut is largely devoid of cyanide at the same time that cyanide is present in the foregut. These observations show that cyanide depletion increases with gut residency time. Although the results of our three studies indicate that the fecal pellets of tent caterpillars contain relatively small amounts of cyanide, the cyanide load of fecal pellets may vary more widely in caterpillars under field conditions. As shown by Casey et al. (1988), the rate at which food transits the caterpillar's gut and the rate of fecal pellet production are temperature-dependant processes. On cool, cloudy days, caterpillars process food slowly, and the bolus remains in the gut for an extended period. When the sun is available, the caterpillars bask and may achieve body temperatures approaching 40°C (Casey et al., 1988; Joos et al., 1988), causing food to transit the gut rapidly. The effect of such rapid transit on the cyanide content of egested material is unknown.

The mechanism by which the eastern tent caterpillar avoids the toxic effects of cyanide is unknown, but three mechanisms have been proposed for caterpillars in general. Rhodanese (thiosufate sulfur transferase) is commonly employed by animals to catalyze the conversion of HCN to thiocyanate (Conn, 1979). Rhodanese is widely distributed in insects, but its role in cyanide detoxification may be marginal to its role as a sulfur donor to proteins (Beesley et al., 1985). Although rhodanese occurs in only trace quantities in the larvae of *M. neustrium*, the

caterpillars are able to consume the highly cyanogenic leaves of *P. laurocerasus* (Parsons and Rothschild, 1964), suggesting that some other detoxification mechanism is involved. Studies by Witthohn and Naumann (1987) and Meyers and Ahmad (1991) indicate that detoxification of cyanide by L-3-cyanoalanine synthase may be the primary mechanism employed by insects. L-3-Cyanoalanine was found to occur in the adults of *M. neustrium* (Witthohn and Naumann, 1987), but it has not been looked for in the larvae of any species of tent caterpillar. Recently, Engler et al. (2000) reported that a species of tropical caterpillar metabolizes leaf cyanogens, preventing the release of cyanide in the body. The results of our study and that of Peterson et al. (1987), showing cyanide in the defensive regurgitations of the caterpillar, however, make sequestration an unlikely possibility in the tent caterpillar.

Eastern tent caterpillars leave their natal tree under two conditions—following premature defoliation of the host and when fully grown and ready to seek pupation sites (Fitzgerald, 1995). In the first instance, the caterpillars exhibit great reluctance to move off the host tree onto the ground. They repeatedly comb the branches of the tree in a fruitless search for food that may last for several days (Snodgrass, 1922; Fitzgerald, 1995). When the caterpillars finally leave the tree, their guts are almost certainly devoid of food. In the second instance, fully grown caterpillars that disperse in the sixth instar may carry cyanide-ladened food in their foreguts but, as our study shows, the total cyanide load is likely to be small.

Small amounts of cyanide survive transit through the gut of the caterpillar and are encapsulated in the fecal pellet. The fecal pellet undergoes dehydration and compaction in the rectum, so on a per-unit-mass basis, pellets are likely to contain more HCN than the midgut bolus itself. After deposition, the pellet dries rapidly and, again, on a per-unit-mass basis, its cyanide concentration increases. In addition to the HCN found in the fecal pellets as detected by our chemical assays, it is possible that the pellets may also contain unhydrolyzed cyanogenic glycoside, which may add to their toxicity in animals that have the ability to hydrolyze the glycosides after ingesting the pellet (Conn, 1979).

Our study shows that as leaf material passes from the tree to the caterpillar to the detritus pool, the cyanide content of the material is greatly reduced but not entirely eliminated. Although many organisms have some capacity to detoxify cyanide (Westley, 1973), it is not known whether the small amounts of cyanide that remain in the fecal pellet have any influence on consumers or detritivores. While the amount of cyanide per fecal pellet is small, colonies produce large quantities of pellets: approximately half of the energy ingested is voided as fecal pellets (Fitzgerald, 1995). However, the production of fecal pellets is likely to be relatively small if caterpillars abandon prematurely defoliated trees before achieving the last instar. A study of the related forest tent caterpillar (*M. disstria*), which may also feed on *P. serotina*, showed that approximately 82% of all the aspen

foliage consumed during the lifetime of the caterpillar was consumed by the last instar.

The oral LD_{50} of HCN for the mouse is 3.7 mg/kg (Sax and Lewis, 1989). In contrast to the small residue of cyanide in the fecal pellet, our study indicates that newly fed caterpillars may carry cyanide in their foreguts in quantities sufficient to poison small predators that are unable to neutralize the toxin. The most likely predators of eastern tent caterpillars are birds nesting in areas infested with colonies of the insect. Numerous studies have shown, however, that with few exceptions, birds reject tent caterpillars as a food source, after initially sampling them, because of their hairiness (Fitzgerald, 1995), but it remains a possibility that some find their way to nestlings. Unlike avian predators, small mammals, frogs, and toads that prey on insects are unlikely to climb trees to search for prey, but a naive predator might attack a sixth-instar caterpillar that is dispersing over the ground with potentially negative consequences. Small fish, too, might be poisoned from consuming recently fed tent caterpillars that have dropped into the water from nests in overhanging trees. Nothing is currently known of the fate of the fecal pellet after it reaches the ground, and it remains to be determined if the residual cyanide it contains has any significance for the small detritivores and decomposers that eventually attack it.

In addition to fecal pellets, colonies of caterpillars also send fragments of partially consumed leaves to the ground, but the quantities involved have not yet been measured. Moreover, it remains to be determined whether animals grazing under infested trees would consume the fallout of leaf fragments and fecal pellets along with grasses, and, if so, what influence the small quantities involved might have on them. Both Morse and Howard (1898) and Smeathers et al. (1978) found greatly reduced quantities of cyanide in dried leaves compared with fresh leaves. There have also been reports of tent caterpillars and fecal pellets dropping into water troughs, and it remains a possibility that some small amount of cyanide might be transferred to animals that drink from such troughs. Larger amounts of cyanide could potentially be transferred to grazers that ingested recently fed, dispersing caterpillars. Although it would seem most likely that grazers would reject the hairy and bitter caterpillars, the guts of some horses from areas experiencing MRLS were shown to contained the remains of caterpillars (T. Tobin, personal communication). Compared to the small amount of cyanide likely to be found in caterpillars moving over the ground, in the fallout under infested trees, or in water troughs visited by tent caterpillars, the highly cyanogenic leaves of cherry trees, or their small seedlings secreted among grasses, may pose a much greater potential threat to grazers and browsers.

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INFLUENCE OF FLAVOR ON GOAT FEEDING PREFERENCES

G. DE ROSA,[1,*] L. MOIO,[2] F. NAPOLITANO,[3] F. GRASSO,[1] L. GUBITOSI,[1] and A. BORDI[1]

[1]*Dipartimento di Scienze zootecniche e Ispezione degli alimenti*

[2]*Dipartimento di Scienza degli Alimenti*
Università degli Studi di Napoli "Federico II"
Via Università 133, 80055 Portici (NA), Italy

[3]*Dipartimento di Scienze delle Produzioni Animali*
Università degli Studi della Basilicata
Via N. Sauro 85, 85100 Potenza, Italy

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Abstract—Goat feeding preferences for straw pellets flavored with ryegrass (*Lolium perenne*, cv. Belida) or white clover (*Trifolium repens*, cv. Huia) aromatic extracts, obtained by means of a cold aromatic extraction method, were assessed with cafeteria trials. Prior to the trials, odor differences between the two plant species, the two aromatic extracts, and the straw pellets sprayed with the two aromatic extracts were verified using sensory analysis performed by 30 human panelists. Since odor differences observed among fresh samples were still detectable in aromatic extracts and moistened pellets, the extraction method was considered effective in reproducing plant odors. Straw pellets sprayed with either distilled water (W) or ryegrass (R) or clover (C) aromatic extracts were used to assess flavor preferences of 12 female Rossa Mediterranea goats. Sprayed pellet preference was evaluated in two sessions conducted in two consecutive weeks. Each session consisted of three two-choice presentations performed on three consecutive days. In both sessions, food intake, proportion of food intake, time spent feeding, and proportion of time spent feeding were significantly affected by pellet type ($0.05 > P > 0.001$). In particular, straw pellets sprayed with ryegrass extract were highly selected compared to those sprayed with clover ($0.01 > P > 0.001$) or water ($0.01 > P > 0.001$). In addition, in the second session, the clover extract was preferred to distilled water ($0.05 > P > 0.01$). The results of this study gave two main indications: first, goat selectivity for ryegrass against clover was consistent even when straw pellets sprayed with odors of these plants were offered, and secondly, the addition of aromatic extracts to straw pellets increased the preference for pellets.

*To whom correspondence should be addressed.

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Key Words—Ryegrass, clover, goats, food preferences, flavor, aromatic extracts, cafeteria trials.

INTRODUCTION

In temperate grassland, goats actively select grasses over legumes. This results in increased clover content in goat-grazed pastures as compared with those grazed by sheep (Radcliffe and Francis, 1988; Bown et al., 1989; del Pozo and Wright, 1995). Lambs grazing after goats consume diets with higher clover content and show higher growth rates than those grazing after sheep (del Pozo and Wright, 1995). Goat selectivity for grasses as opposed to legumes is also consistent in short-term trials (De Rosa et al., 1997). Although levels of non-structural carbohydrates are equivalent between legumes and grasses, legumes contain a much lower proportion of water-soluble carbohydrate (Smith, 1973; Osbourn, 1980). The different dietary preferences observed in sheep and goats may be due to the fact that goats ingest plants with high non-structural carbohydrate content (Fedele et al., 1993), whereas plants with higher levels of secondary metabolites such as tannins, alkaloids, terpenes, and cyanogenic glycosides (e.g., legumes) are disliked (Narjisse, 1991; Morand-Fehr et al., 1993; Narjisse et al., 1996).

The forage preference is considered to be a function of taste, odor, texture (forage flavor), and postingestive effects on the animal (Provenza, 1995, 1996). Animals acquire preferences for nutritious foods and dislike foods with low nutrient content or high toxins levels (Provenza, 1995, 1996).

Goats learn to avoid condensed tannins from the current season's twigs of blackbrush (*Coleogyne ramosissima*) by associating the flavor of foods containing condensed tannins with aversive postingestive effects (Provenza et al., 1990). Sheep and goats appear to discriminate against monoterpenoid odor and taste, respectively, but this possibility has not been fully ascertained (Narjisse et al., 1996). Sheep regulate intake according to the flavor concentration of the feed, when flavor and toxicity are strongly correlated (Launchbaugh et al., 1993). In contrast, when they are not associated, animal intake relied on the maximum dose of toxin they had ingested (Launchbaugh et al., 1993). Sheep also more readily eat an unfamiliar feed associated with a familiar flavor or odor, whereas they avoid it when presented with a novel odor (Van Tien et al., 1998). Moreover, it has been shown that in sheep, hay palatability may be modified using different chemical compounds. The intake of wheaten hay was increased by the addition of butyric acid and monosodium glutamate, whereas it was reduced by MgO (Gherardi and Black, 1991; Gherardi et al., 1991). These results indicate that the flavor of the forage may play an important role in determining herbivore feeding preferences.

The present study aimed to evaluate the importance of flavor on feeding preferences of goats. We developed a cold aromatic extraction method for ryegrass

(*Lolium perenne*, cv. Belida) and white clover (*Trifolium repens*, cv. Huia) herbage that was effective in reproducing the corresponding plant's odor characteristics. Feeding preferences can be assessed by either long-term trials, comparing the proportion of a plant species in the diet as against its proportion in the sward, or cafeteria trials, directly testing preferences using two-choice presentations (Gordon, 1995). Goat feeding preferences for the aromatic extracts sprayed on straw pellets were assessed using cafeteria trials. The plants were chosen according to their different palatability as shown by Fedele et al. (1993) in field trials conducted in a Mediterranean grazing environment and by De Rosa et al. (1997) in cafeteria trials, who noted that ryegrass was highly selected, whereas clover was disliked by goats.

METHODS AND MATERIALS

Odor Differences between Ryegrass and White Clover. Prior to preparation of aromatic extracts from ryegrass and white clover, odor differences between the two plant species were verified using sensory analysis. Thirty human panelists, (students and staff members of the Department of Animal Production, University of Naples), were selected on the basis of their availability and motivation. Triangle tests were performed, offering each member two sets of three samples of herbage in the vegetative stage harvested just before use and chopped into 4- to 6-cm pieces. The weight of each sample was 30 g. Two samples were alike and one was different within each triangle test. Samples were presented in Teflon bags that lay on a layer of ice placed in Pyrex beakers to avoid olfactory bias and visual discrimination. Panel members were asked to identify the odd sample. Triangle tests were performed in a 25-m^2 room where the panel members were individually introduced to the person conducting the test. Three sessions of the triangle test were performed per panelist.

Preparation of Aqueous Aromatic Extracts. Samples from the two plant species were harvested and immediately frozen in liquid nitrogen in order to minimize degradation and/or transformation of odorous molecules. Frozen plants were powdered in liquid nitrogen and added to distilled water at 50, 100, or 200 g/liter. Powder and water were mixed for 48 hr at 4°C. Subsequently, the aqueous solution containing volatile components was centrifuged for 10 min at 7000 rpm and stored at −80°C until use. A sensory test was conducted to determine the concentration of the aromatic extract that most resembled the corresponding plant smell. Extracts were thawed overnight at 4°C and introduced in 50-ml flasks wrapped with aluminum foil. Human panelists were asked to associate, by sniffing, a series of different concentrations of aromatic extract (5, 10, and 20%) to the original plant species. For each plant species, panelists received three samples of extracts and a sample of corresponding herbage. Three sessions of this test (the similarity test) were performed. The panelists chose the 10% powder concentration for

both ryegrass and clover as most similar to the corresponding plant species (see Results). Therefore, this concentration was used for the successive experimental steps.

Subsequently, three triangle test sessions for aqueous aromatic extracts of ryegrass and clover were performed as described above to test whether odor differences between the two plant species were consistent in the corresponding aromatic aqueous solutions after the cold extraction.

Preparation of Samples Offered to Animals. Straw pellets are characterized by a low-intensity odor that is cosidered unlikely to interfere with the volatile components of an aqueous solution. Straw pellets are also known to be disliked by goats, so that any increase or decrease in consumption may be attributed to the effect of extract addition. Thus, the aromatic extracts to be offered to the goats were incorporated into straw pellets. Three sessions of triangular tests where the straw pellets were sprayed with ryegrass or clover aromatic extracts (50 ml of aqueous solution for 200 g of straw pellet) were performed.

Experimental Design. Twelve female Rossa Mediterranea goats were used in this experiment. The age and weight of the animals were 2–3 years and 35–45 kg, respectively. The goats were not pregnant or lactating, and all had previous experience of consuming both plant species.

The goats were equally divided into two groups and group-housed in two straw-bedded pens (4 $\times$ 2 m) with water available *ad libitum*. The animals were exposed to a three-week preliminary phase, during which they were offered straw pellets ad libitum in plastic baskets (50 $\times$ 34 $\times$ 15 cm) from 09:00 to 14:00 hr and, subsequently, mixed grass hay *ad libitum*.

In a subsequent two-week training phase, the animals were moved once a day into individual test pens (1.2 $\times$ 2 m) for 10 min where they received approximately 800 g of straw pellets in four plastic baskets placed in a wooden trough located 30 cm above floor level. An observer sat in front of each individual test pen to acclimate the animals to human presence. The goats in the adjacent test pens could not see each other. Thereafter, the animals were moved to the home pen where *ad libitum* mixed grass hay was offered. In the last two days of the training phase, straw pellets were sprayed with distilled water (50 ml/200 g).

Cafeteria Trials. During the preference tests, straw pellets sprayed (50 ml/200 g) with either distilled water (W) or ryegrass (R) or clover (C) aromatic extracts were used to assess goat flavor preference. The pellets were sprayed immediately prior to the start of the preference tests.

For each goat, sprayed pellet preference was evaluated in two sessions performed in two consecutive weeks. Each session consisted of three two-choice presentations conducted over three consecutive days. Two-choice presentations started at 09:00 hr and never continued past 12:00 hr. Five minutes before each presentation, the animals were moved to the individual test pens. The animals were offered two samples (sprayed pellets) simultaneously for 3 min in four adjacent

baskets (200 g/basket) filling all the through front. Two consecutive baskets contained sample 1 and two contained sample 2. On test days, each pair of samples (ryegrass–water, clover–water, and ryegrass–clover) was offered to four animals to avoid day effect. Three goats from the same group were simultaneously tested, always at the same time of day, with each animal getting a different pairing. Goats were used for only one two-choice presentation a day. Samples were assessed with the same frequency in each spatial (left–right) and temporal position (ryegrass–water, clover–water, ryegrass–clover), thereby minimizing the effects of position habits and order of presentation. Samples were weighed before and after each presentation in order to determine the total intake using an electronic digital scale. Six control baskets (two for each sample) were used to estimate the evaporative weight losses and the total intake was corrected accordingly. During the two-choice presentation an observer recorded feeding time (prehension time + mastication time) for each sample. For each session, the preference of the animals for each sample was determined by using the averages of time spent feeding, food intake, proportion of time spent feeding (time spent feeding on one sample/total feeding time), and proportion of food intake (intake from one sample/total intake) recorded in all the two-choice presentations where the sample was present (Morand-Fehr et al., 1987). After the two-choice presentations, the animal were moved to the home pen and fed with mixed grass hay *ad libitum*.

Statistical Analyses. Data were analyzed with a statistical package for social sciences (SPSS-PC+, version 3.1) (Norusis, 1989). Data obtained from human sensory evaluation were analyzed with the χ^2 one-sample test, where the expected frequencies of the odd samples (triangle tests) and of each concentration of aromatic extract (similarity tests) were 33.3%.

For the cafeteria data, the goat was used as the experimental unit. For each session of cafeteria trial, the variables collected (time spent feeding, food intake, proportion of time spent feeding, and proportion of food intake) were analyzed using the Friedman two-way analysis of variance by ranks test with pellet type (R, C, and W) as factor. When significant effects were recorded, the differences among the means were evaluated by the Wilcoxon matched-pairs signed-ranks test. The latter was also used to test session effect for each type of pellet.

RESULTS

Validation of Extraction Technique. Triangular tests performed on the two plant species, their aromatic extracts, and moistened pellets showed significant differences between clover and ryegrass odors ($P < 0.001$) (Table 1). Since odor differences observed between fresh samples were still detectable in aromatic extracts and moistened pellets, the extraction method can be considered effective in reproducing plant odors.

TABLE 1. DEGREE OF DISCRIMINATION OBSERVED DURING TRIANGLE TESTS OF RYEGRASS AND CLOVER OFFERED AS HERBAGE, AROMATIC EXTRACTS, AND SPRAYED STRAW PELLETS TO 30 PANELISTS

Sample	Comparison	Session	Proportion of correct responses	Significance[a] (P)
		I	0.83	<0.001
Herbage	ryegrass–clover	II	0.83	<0.001
		III	0.87	<0.001
		I	0.87	<0.001
Aromatic extract	ryegrass–clover	II	0.85	<0.001
		III	0.73	<0.001
		I	0.72	<0.001
Sprayed pellets	ryegrass–clover	II	0.75	<0.001
		III	0.80	<0.001

[a] χ^2 one-sample test; $df = 1$.

Similarity tests were conducted to evaluate the concentration of aqueous extracts that presented the odor closest to the corresponding plant (Table 2). Results indicated that a 10% powder concentration could be considered to be the most similar to the corresponding plant, since the choice of assessors for this concentration over the three sessions was 55.6% and 55.7% for ryegrass and clover, respectively ($\chi^2 = 7.02$, $df = 2$, $P < 0.05$ and $\chi^2 = 8.6$, $df = 2$, $P < 0.05$, respectively). Therefore, this concentration was used in cafeteria trials.

Cafeteria Trials. Food intake (Figure 1) was significantly affected by pellet type in both sessions ($\chi^2 = 6.5$, $df = 2$, $P < 0.05$ and $\chi^2 = 13.54$, $df = 2$, $P < 0.001$, respectively). In the first session, R consumption was higher than C and W

TABLE 2. SIMILARITY OBSERVED BY 30 PANELISTS BETWEEN HERBAGE AND CORRESPONDING AROMATIC EXTRACT

Herbage	Concentration of aromatic extract (%)	Proportion of responses per session[a]		
		I	II	III
	5	0.20	0.13	0.20
Ryegrass	10	0.53	0.54	0.60
	20	0.27	0.33	0.20
χ^2		5.6*	7.2*	9.6**
	5	0.27	0.33	0.30
Clover	10	0.60	0.54	0.57
	20	0.13	0.13	0.13
χ^2		10.4**	7.2*	8.6*

[a] χ^2 one-sample test; $df = 2$; *$P < 0.05$; **$P < 0.01$.

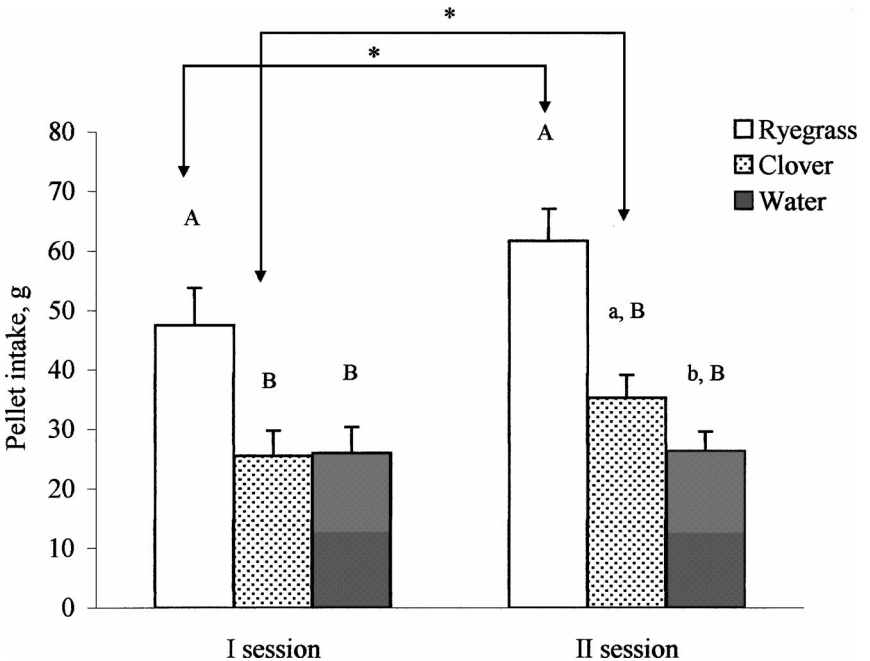


FIG. 1. Goat intake (mean + SE) of straw pellets sprayed with either distilled water or ryegrass or clover aromatic extracts in the two session cafeteria trial. A, B: means within sessions followed by a different letter are different at $P < 0.001$. a, b: means within sessions followed by a different letter are different at $P < 0.05$. $*$ means between sessions are different at $P < 0.05$.

($P < 0.001$). There was no difference between C and W. In the second session, goats showed a higher intake for R than for C and W ($P < 0.001$), as well as a higher ingestion of C than W ($P < 0.05$). A comparison between the two sessions showed higher intakes for R and C in the second trial ($P < 0.05$), but no differences between trials for W. Similar results were observed for the proportion of food intake except for the differences between trials (Figure 2).

For both sessions a significant effect of pellet type on time spent feeding ($\chi^2 = 6.5$, $df = 2$, $P < 0.05$ and $\chi^2 = 17.17$, $df = 2$, $P < 0.001$, respectively; Figure 3) was found. In fact, in the first session, the animals spent more time eating R than C and W ($P < 0.01$). There were no differences observed between C and W. In the second session, time spent feeding on R was higher than that spent on C and W ($P < 0.01$), whereas this variable was lower for W than for C ($P < 0.01$). A comparison between the two sessions showed higher levels of time spent feeding on W in the first session ($P < 0.05$). Accordingly, the proportion of time spent feeding showed a similar pattern (Figure 4).

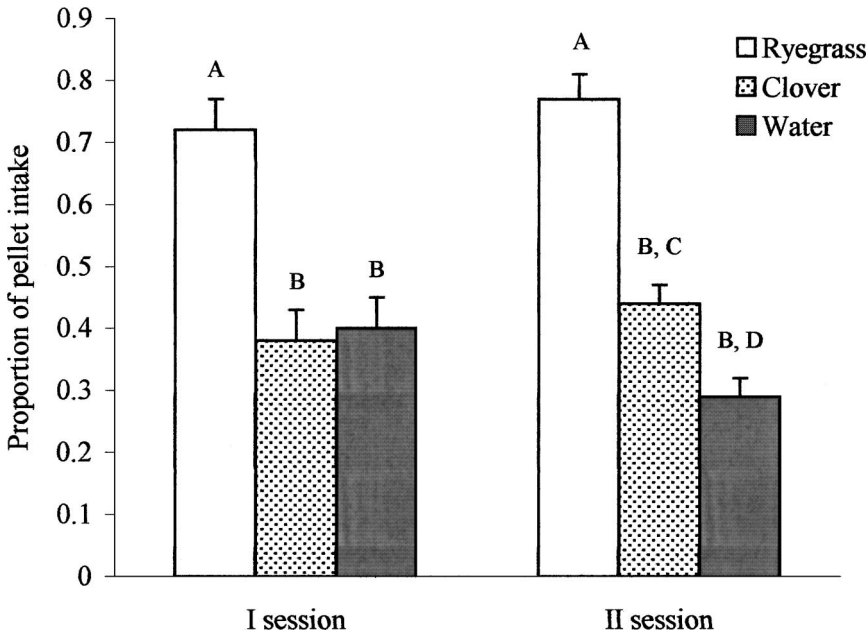


FIG. 2. Proportion of goat intake (mean + SE) of straw pellets sprayed with either distilled water or ryegrass or clover aromatic extracts in the two session cafeteria trial. A, B: means within sessions followed by a different letter are different at $P < 0.001$. C, D: means within sessions followed by a different letter are different at $P < 0.01$.

DISCUSSION

In this study, plant physical characteristics were removed by transferring the odorous molecules from ryegrass and clover to aqueous solutions. However, the aroma of the original sample extract may change during the extraction process and does not necessarily reproduce the characteristics of the original sample from which it was obtained (Moio et al., 1995). If such an odor distortion occurs during the preparation of extract, the aromatic aqueous phase offered to the animals may exhibit olfactory properties different from those of the original plant. Therefore, the first objective of this study was to develop an extraction procedure yielding extracts with odors close to those of the original plant. The results of sensory analysis indicate that the cold aromatic extraction technique from ryegrass and white clover may be effective in reproducing the corresponding plant odor characteristics. The importance of using a low temperature during the extraction process to attain a good representation of the extracts before sensory analysis has also been reported for wine (Moio et al., 1995), cheese, and tomato (Etievant et al., 1995).

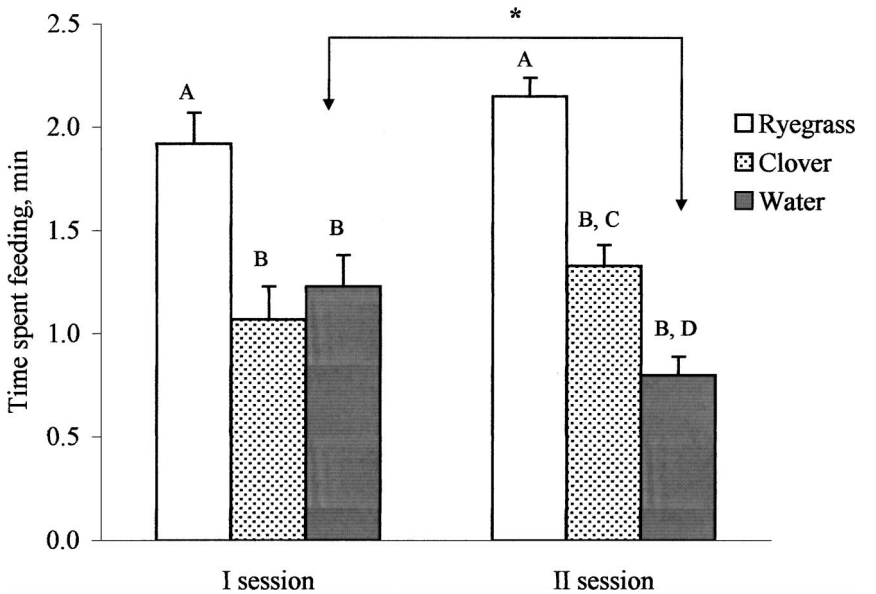


FIG. 3. Time spent feeding (mean + SE) by goats on pellets sprayed with either distilled water or ryegrass or clover aromatic extracts in the two session cafeteria trial. A, B and C, D: means within sessions followed by a different letter are different at $P < 0.01$. $*$ means between sessions are different at $P < 0.05$.

The results obtained in the cafeteria trials with the four dependent variables (time spent feeding, food intake, proportion of time spent feeding, and proportion of food intake) were generally similar. This study indicates that the addition of aromatic extract (ryegrass or clover) to straw pellets usually increased the preference for pellets. In particular, the intake of straw pellets sprayed with ryegrass extract was higher than those sprayed with water in both sessions, whereas for the clover extract this was observed only in the second session. These findings are in agreement with Provenza et al. (1996), who suggested that offering lambs the same food in different flavors influences food intake, as does offering different foods of similar or different nutritional value.

In field experiments, it has been found that ryegrass and white clover swards grazed by goats have more clover than those grazed by sheep (Penning et al., 1996; del Pozo et al., 1998). In a Mediterranean grazing environment, where the vegetation was exclusively herbaceous, Fedele et al. (1993) found that goats preferred grasses and had little interest in forbs and legumes. A preference for grasses over legumes was also confirmed in short-term trials (De Rosa et al., 1997). It has been suggested that goats prefer plants with high non-structural carbohydrate content (Fedele et al., 1993), whereas plants with higher levels of secondary metabolites,

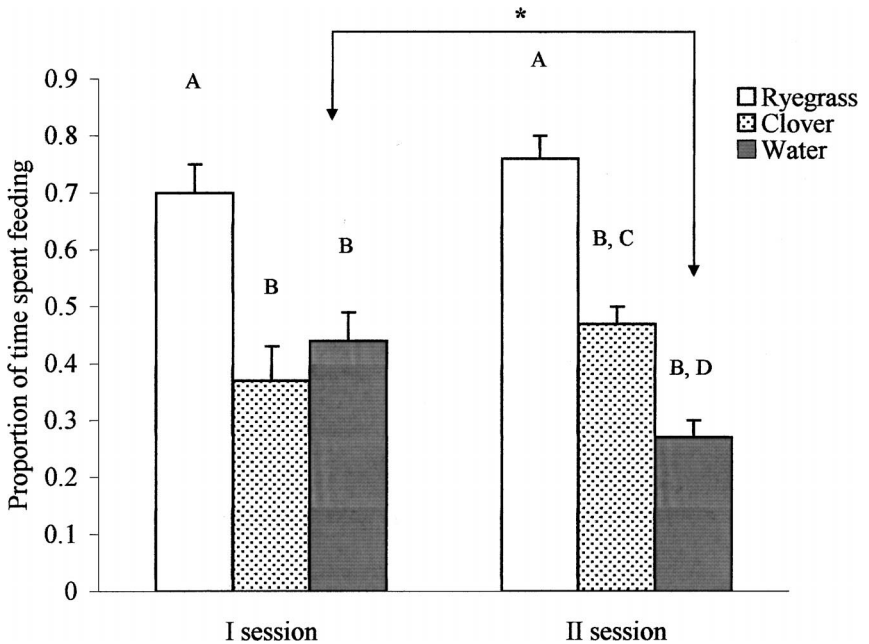


FIG. 4. Proportion of time spent feeding (mean + SE) by goats on straw pellets sprayed with either distilled water or ryegrass or clover aromatic extracts in the two session cafeteria trial. A, B and C, D: means within sessions followed by a different letter are different at $P < 0.01$. $*$ means between sessions are different at $P < 0.05$.

which are considered protective agents for plants against herbivores and pathogens (Provenza et al., 1988) such as tannins, terpenes, alkaloids, cyanogenic glycosides (e.g., legumes) are markedly disliked by goats (Narjisse, 1991; Morand–Fehr et al., 1993). In fact, removal of surface compounds with organic solvents increased tarbush (*Flourensia cernua*) intake by sheep (Estell et al., 1994), and terpenes on the leaf surface were related to diet preference (Estell et al., 1996). Accordingly, diet selection of goats was inversely correlated with tannins (Provenza and Malechek, 1984; Provenza et al., 1990). Goats were also able to taste monoterpenes (Narjisse et al., 1996) in pellets, and a positive (camphor) or negative (α-pinene, limonene) correlation between the content of these monoterpenes with juniper ingestion has been found (Riddle et al., 1996). Conversely, in lambs a detrimental effect of both camphor and α-pinene concentration on pellet intake has been reported (Estell et al., 1998). These findings are consistent with our results where the preference was higher for ryegrass extract than for clover, although the nutritional value of the two pellets was similar and the factor of plant physiological characteristics was removed. Therefore, only sensory cues (odor and taste) could be used by goats for

pellet discrimination, thus confirming that food flavor may play an important role in affecting goat preferences.

Food preferences depend on the functional interrelationship between flavor and postingestive effects of nutrients (Provenza et al., 1996). Animals discriminate among foods using taste, odor, and sight, which are a source of hedonic sensations (Provenza, 1996). They acquire preference for nutrients and for high levels of rapidly digestible nutrients and an aversion for toxins and foods deficient in specific nutrients (Provenza, 1995, 1996). It can be argued that in the present study goat selection was not based on postingestive effects, since short-term trials were performed. Thus, the data may reflect only the initial response of goats to the flavors of ryegrass and clover. The animals were exposed to these flavors after one week and the preference for ryegrass over clover was confirmed. The animals used in this study had experienced grazing in open rangeland, where they may have learned to associate a particular flavor with negative (or positive) postingestive effects in previous grazing activity. This hypothesis is supported by experimental evidence that young mammalian herbivores can still associate, even after as long as three years, negative (Burritt and Provenza, 1990; Distel and Provenza, 1991) or positive associations (Squibb et al., 1990; Distel and Provenza, 1991) with specific foods. It is likely that the goats generalized preferences and aversions to the flavored straw pellets on the basis of their past experiences with straw, clover, and ryegrass. Flavor generalizations have been demonstrated for food preferences (Ralphs et al., 1995) and food aversions (Launchbaugh and Provenza, 1993). This is especially likely to be the case given the limited amount of time the goats were tested. With more time and greater intake of the pellets, the postingestive effects of straw would likely have played a greater role in influencing food selection (Lawler et al., 1999; Villalba and Provenza, 2000). In addition, during the cafeteria trials, the goats ate limited amounts of the straw pellets, less than 50 g of each flavored pellet, and they were offered the foods briefly (3 min) on only six occasions. Food items that make up such a small part of the daily ration have relatively little postingestive influence on food preference (Villalba and Provenza, 2000).

Results from this study show that goat selectivity for ryegrass as opposed to clover is consistent, even when straw pellets sprayed with odors of these plants are offered. Further studies using gas chromatography–sniffing and gas chromatography–spectrometry analysis to determine the aromatic compounds responsible for grass–legume discrimination and preference are the next steps, because a better understanding of the mechanisms underlying food preferences may lead to strategies for altering diet selection.

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SPECIFIC ATTRACTION OF FIG-POLLINATING WASPS: ROLE OF VOLATILE COMPOUNDS RELEASED BY TROPICAL FIGS

LAURE GRISON-PIGÉ,[1] JEAN-MARIE BESSIÈRE,[2] and MARTINE HOSSAERT-MCKEY[1,*]

[1]*Centre d'Ecologie Fonctionnelle et Evolutive-CNRS*
1919 route de Mende
34293 Montpellier Cedex 5, France

[2]*Laboratoire de chimie appliquée*
Ecole Nationale Supérieure de Chimie de Montpellier
8 rue de l'Ecole Normale
34296 Montpellier Cedex 5, France

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Abstract—Floral scents often act as pollinator attractants. In the case of obligate and specific plant–pollinator relationships, the role of floral signals may be crucial in allowing the encounter of the partners. About 750 *Ficus* species (Moraceae) are involved in such interactions, each with a distinct species of pollinating wasp (Chalcidoidea, Agaonidae). Several species have been shown to release volatile compounds, but their role in pollinator attraction has rarely been simultaneously tested. We investigated the floral scents of four tropical fig species and combined chemical analysis with biological tests of stimulation of insects. Pollinators of three species were stimulated by the odor of their associated fig species and generally not by the odor of another species. The fourth actually comprised two distinct varieties. The main compound was often a different one in each species. Floral blends of different species always shared compounds, but ratios of these compounds varied among species.

Key Words—*Ficus*, Moraceae, volatile compounds, mutualism, fig wasps, bioassays.

INTRODUCTION

Floral scents are supposed to have evolved from herbivore deterrents (Pellmyr and Thien, 1986) and to act as pollinator attractants in numerous species (Williams

*To whom correspondence should be addressed.

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and Dodson, 1971; Groth et al., 1987; Dobson, 1994). Their chemical composition has often been studied (Pellmyr et al., 1991; Bergström et al., 1992; Moya and Ackerman, 1993; Pichersky et al., 1994; Bestmann et al., 1997), but the role of these odors in pollinator attraction has rarely been tested (but see Roy and Raguso, 1997). *Ficus* species (Moraceae) interact with their pollinators (Chalcidoidea, Agaonidae) in a species-specific mutualism, where each partner needs the other to reproduce (Janzen, 1979; Wiebes, 1979). Each pollinator species is associated with a single *Ficus* species. In this obligate and specific relationship, encounter with the partner is a crucial step, and recognition signals, thus, play an important role. *Ficus* species mainly live in tropical regions, and many species can cooccur in the same forest. In these regions, the density of individual species is often quite low. Therefore, signals emitted by a species must be efficient at long distances and specific, to allow the attraction of the associated pollinator. In several of the 750 *Ficus* species, figs have been shown to release volatiles when they are receptive (i.e., at the stage when pollination can occur) (Barker, 1985; Van Noort et al., 1989; Ware et al., 1993; Hossaert-McKey et al., 1994; Ware and Compton, 1994; Harrison, 1996; Gibernau et al., 1998; Grison et al., 1999). Biological tests have been performed with three species (*F. burtt-davyi*, *F. thonningii*, and *F. carica*), confirming for these species the role of fig scent in insect attraction (Van Noort et al., 1989; Hossaert-McKey et al., 1994; Ware and Compton, 1994; Gibernau et al., 1998), either with figs enclosed in bags (in these cases compounds were not identified), or with pentane extracts. In this work, we used a combination of biological tests with volatile compounds present in the fig headspace and performed chemical analysis of this blend. To our knowledge, this is the first time that tests have been performed on fig pollinators with volatile blends of known composition. Our aim was to investigate, in several tropical species, the role of fig scent in pollinator stimulation and in the specificity of the fig–fig wasp interaction.

METHODS AND MATERIALS

Study Species and Site. We studied four tropical fig species in Brunei (on the island of Borneo). For each species, odor released by receptive figs was collected and analyzed, and biological tests were performed. As often as possible (depending on wasp availability), tests were also done with pollinators of other fig species.

Figs are urn-shaped inflorescences, closed by imbricate covering bracts, forming the ostiole. Half of all fig species are monoecious, the other half gynodioecious. In monoecious species, when the inflorescence is receptive, it attracts females of a specific agaonid species, which enter the fig through the ostiole, lay eggs, and pollinate. Seeds and pollinators develop inside the fig. Several weeks later, male flowers inside the fig produce pollen and, at the same time, pollinators exit their galls and mate inside the fig. Female wasps will then leave the fig, loaded with

pollen grains. During this phase, they have to find a new fig of their associated species, in the receptive phase, to lay eggs. Because of the synchrony of figs on the same tree, pollinators have to fly away from their natal tree in order to be able to find a receptive fig. Their short life-span (2-3 days) (Kjellberg et al., 1988) makes this step critical for the accomplishment of their life cycle [see Janzen (1979) and Wiebes (1979) for more details on the fig–fig wasp cycle]. In gynodioecious species, female trees produce seeds, and hermaphrodite trees (which are functionally male) produce pollen and pollinators (Janzen, 1979; Kjellberg et al., 1987). In these species, as well, upon their exit from the natal fig, female wasps must fly away to find a new receptive fig. They may visit figs on either male or female trees.

Mature and secondary rainforests in Brunei contain 80 *Ficus* species (Corner, 1965). This study involved four of them (Table 1). Three are dioecious: *F. fulva*, *F. deltoidea* (both subgenus *Ficus*; associated pollinators: *Blastophaga compacta* and *Wiebesia* sp., respectively), and *F. condensa* (subgenus Sycomorus, associated pollinator: *Ceratosolen constrictus*), and one is monoecious: *F. microcarpa* (subgenus *Urostigma*; associated pollinator *Eupristina verticillata*). *Ficus fulva* and *F. condensa* are small trees, living in secondary forests (often along riverbanks for *F. condensa*) or in forest understorey (Corner, 1965). *Ficus microcarpa*, a banyan tree, is a hemiepiphyte found in the forest canopy or in mangroves. *Ficus deltoidea* can be found in the forest as an epiphyte, but the individuals studied here grow as shrubs in sandy soil.

Collection and Analysis of Volatile Compounds. Volatile compounds released by figs were collected by adsorption–desorption (headspace technique) (Turlings et al., 1991; Grison et al., 1999). Because of the necessity to obtain concentrated extracts, collections were not performed in situ. Branches bearing figs were cut and leaves removed (to prevent dehydration due to transpiration). They were then put in a bag with wet paper sheets during the transportation, which never lasted more than 90 min. For odor collection, branches were enclosed in polyethylene terephthalate (Nalophan) bags, which have been shown not to release volatiles, and put in a water-containing vase. Airflow was maintained through the bag by

TABLE 1. SPECIES AND SITES[a]

Species	Site	Latitude (°N)	Longitude (°E)
F. fulva female	Bandar Seri Begawan (museum and city)	4.9	114.9
	Labi Road	4.7	114.3
F. fulva male	Jalan Muara	5	115
F. condensa male	Bandar Seri Begawan (museum and city)	4.9	114.9
	Jalan Muara	5	115
F. deltoidea male	Tutong	4.7	114.3

[a]All sites were disturbed by human activity.

two pumps. Entrance and exit flow rates (controlled by flowmeters) were 400 and 300 ml/min, respectively. The bags were then pressurized so that pollution by external air was prevented. At the entrance of the bag, air was purified in a charcoal filter. At the exit, it went through a Porapak Q trap (25 mg, 80–100 mesh), where compounds were adsorbed.

Collection lasted 5–8 hr. Empty bags and bags containing branches without figs were used as controls. For each species, several collections were performed, depending on the fig phenology (*F. fulva* female: 10 samples, with figs from 3 trees; *F. fulva* male: 2 samples, with figs from 1 tree; *F. condensa* male: 18 samples, with figs from 9 trees; *F. deltoidea* male: 3 samples, with figs from 3 trees; *F. microcarpa*: 5 samples, with figs from 3 trees). During the period of our study, because of the tree phenology, collections of odors from female figs of *F. condensa* and *F. deltoidea* could not be performed. During the collections, numbers (mean $\pm$ SD) of figs in the sample were: for *F. microcarpa*, 950 $\pm$ 531 (mean $\pm$ SD diameter of figs 5.6 mm $\pm$ 0.6), for *F. fulva*, 415 $\pm$ 220 (diameter 12 mm $\pm$ 1.6), for *F. condensa* 255 $\pm$ 116 (diameter 15 mm $\pm$ 2.2) and for *F. deltoidea* 143 $\pm$ 126 (diameter 11 mm $\pm$ 3.6).

Compounds were eluted from Porapak Q with 150 μl of dichloromethane and stored at -20°C until used for chemical analysis or biological tests. Solutions obtained were either used for biological tests (see below) or combined to form a more concentrated solution (one per species) that was analyzed with a gas chromatograph–mass spectrometer (GC: Hewlett-Packard, MS: HP 5870; FID, column DB-5, 30 m, 0.25 mm ID, film thickness 0.25 μm, carrier gas: helium, oven temperature program: 50–180°C, 3°C/min). Noncombined solutions were not concentrated enough for GC or GC-MS analysis. Percentages of compounds in the scent are given on the basis of the chromatogram obtained. Component identification was based on computer matching with Wiley 138 and NBS 75K libraries and on GC retention times. Retention indices were calculated and compared to those in the literature (Adams, 1989). Unknown compounds were identified with a MS library. *Ficus fulva* male scent was not abundant enough to be concentrated and analyzed by GC-MS, and was entirely reserved for bioassays.

Bioassays. For biological tests, mature figs were collected before wasp emergence and kept in a cloth bag at ambient temperature. Emergence of pollinators occurred the same day or the following one, and experiments took place at that time. A drop (1.5 μl) of solvent (dichloromethane) or volatile sample (compounds collected by the headspace technique, in a solution with dichloromethane) was deposited on the top of a glass marble (15 mm diam.). The size of the marble overlapped with fig size, except for *F. microcarpa*, but the results suggest that the size of the marbles did not matter. After 1 min of solvent evaporation, a newly emerged pollinator was deposited on the marble. The length of time it stayed on the marble was recorded (with a maximum time of 10 min, after which the experiment was stopped; pollinators appeared to make their decision before that time),

as an estimate of the stimulation of the insect by the solvent or solution. The term "stimulation" will be used throughout this text as a synonym for the "arrestment" of the insect on the marble, during the time when it wanders and antennates on the surface of the marble. Pollinators attracted to a fig arrive on it, and then, if they are stimulated, they wander on the surface around the ostiole, sweeping it with their antennae for some time before they enter it. If not stimulated, they generally fly away quickly (Gibernau et al., 1998). Thus, the stage tested was the stimulation of the insect once on the fig (and not the attraction at a distance). For each marble, the solvent and then, with another insect, the volatile sample were tested. These bioassays were done outside to prevent contamination of air by previous experiments and to provide pollinators with appropriate temperature and humidity conditions.

Data were analyzed using Mann-Whitney rank-sum tests. For each species, series of control times were compared to series of assay times. Two-tailed probability values were considered. We used the Genmod procedure from the SAS statistical package (SAS, 1999) to test for the effect of the date of the experiment, and no effect was found (*F. fulva*: $F = 0.27$; $P = 0.95$; *F. condensa*: $F = 0.26$, $P = 0.97$; *F. deltoidea*: $F = 0.02$, $P = 0.88$; *F. microcarpa*: $F = 0.89$, $P = 0.35$). Biological tests performed with different volatile samples of the same species and with the same pollinators were, therefore, analyzed together (series of tests performed at different dates were pooled together). Bioassays were performed with volatile samples from male and female *F. fulva*, and male *F. microcarpa, F. deltoidea*, and *F. condensa*, with their associated pollinators. Cross-tests were also done with samples of volatiles from female *F. fulva*, male *F. microcarpa* and *F. condensa*, and pollinators associated with the other two species.

RESULTS

Bioassays showed that the pollinators remained longer on marbles that had received samples of volatiles from their host species than on controls ($P < 0.01$), for *F. condensa, F. microcarpa*, and *F. fulva* female (Figure 1a). Tests with volatile samples from *F. fulva* male ($P = 0.102$) and *F. deltoidea* ($P = 0.850$) were not significant. Durations of the stay on the marbles were highly variable. For the significant tests, durations (mean $\pm$ SD) on the marbles were 18 sec $\pm$ 57 and 74 sec $\pm$ 124 for the solvent and the samples, respectively. Cross-tests performed with samples of volatiles and pollinators of *F. microcarpa, F. fulva*, and *F. condensa* were not significant, except for the test with samples of volatiles from *F. fulva* female and pollinating wasps associated with *F. microcarpa* ($P = 0.002$) (Figure 1b).

For each species blend, 23–53 peaks were visible on the chromatogram. Among these, 89–100% were identified (for the less abundant compounds, identification was sometimes not possible). The identified volatile compounds released

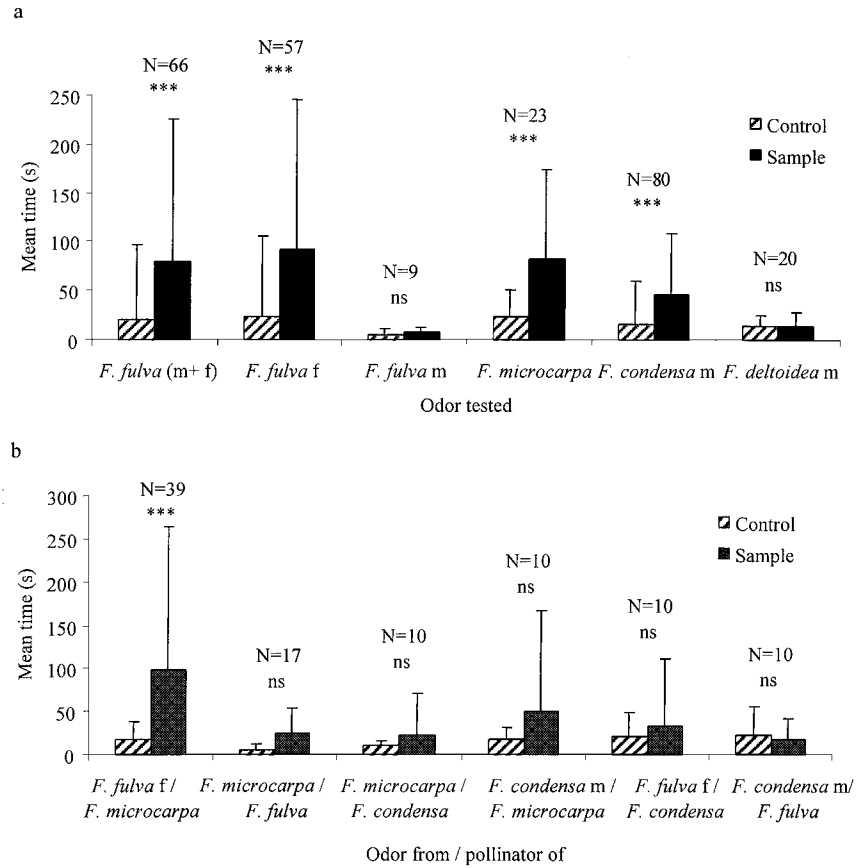


FIG. 1. Species-specific and crossed bioassays were performed with solutions of volatile compounds collected in the headspace of receptive figs of four tropical species (*F. fulva*, *F. condensa*, *F. microcarpa*, and *F. deltoidea*) and with fig pollinating wasps. The results and statistical analyses (Mann-Whitney two-tailed probability) are shown: (a) species-specific tests; (b) cross-tests (odor from one fig species tested with pollinator of another fig species). m: male, f: female, N: number of assays, ns: not significant, $^{***}P < 0.01$. *Note*. maximum time is 10 min.

by receptive figs from the four species mainly belonged to four chemical families: monoterpenes, sesquiterpenes, benzenoids, and aliphatic compounds. Each species blend comprised 9–16 compounds with an abundance greater than 1%, the total abundance of these compounds representing 90–96% of the blend. In each blend, one compound (or few compounds in the case of *F. deltoidea*), most of the time a sesquiterpene, was far more abundant than the others (Figure 2). These main

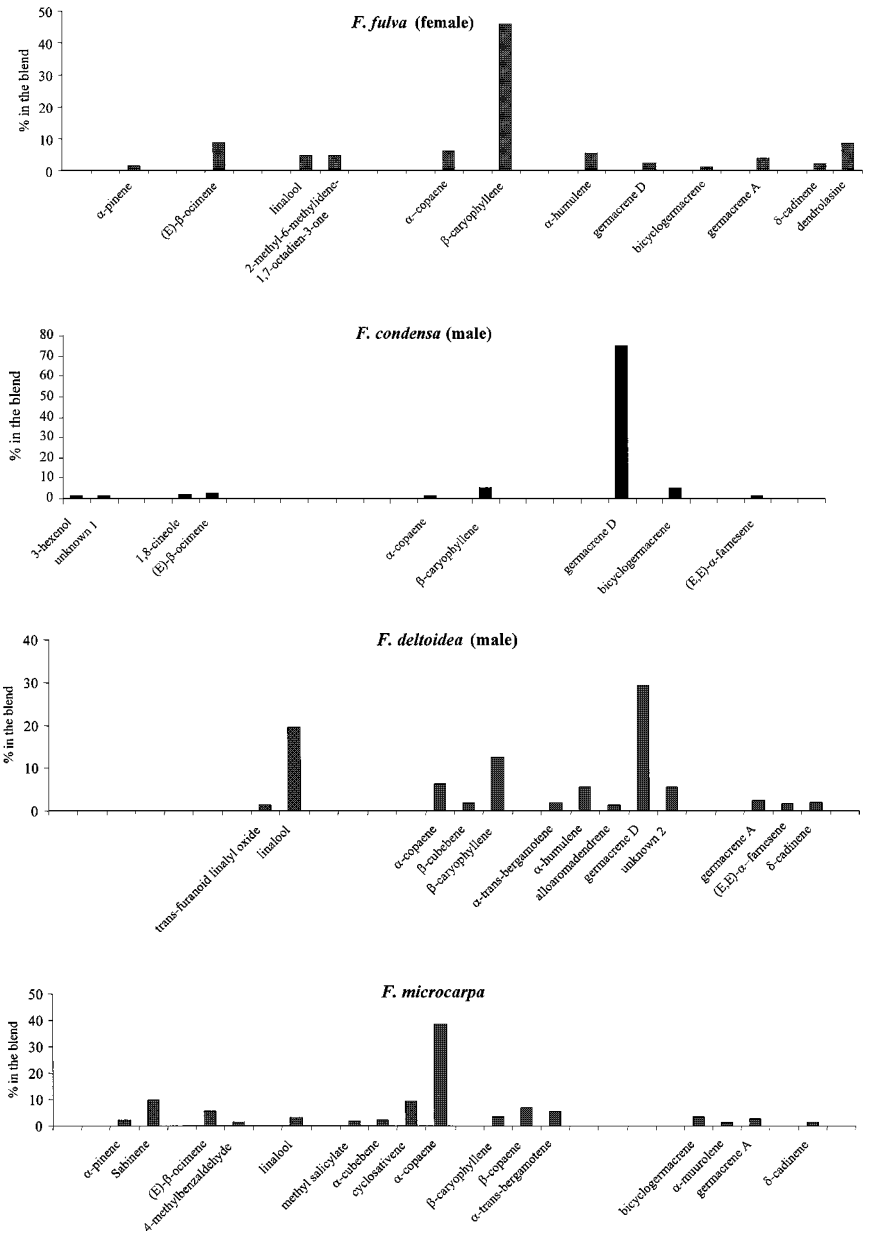


FIG. 2. Chemical composition of the scent present in the headspace of four tropical *Ficus* species: *F. fulva*, *F. condensa*, *F. microcarpa*, and *F. deltoidea*. Only compounds with an abundance >1% are represented.

compounds were β-caryophyllene in the *F. fulva* (female) blend, α-copaene in the *F. microcarpa* blend, and germacrene D in the *F. condensa* blend. In the case of *F. deltoidea* (male), linalool, germacrene D, and β-caryophyllene were the most abundant compounds in the fig fragrance. Most of the compounds were shared by two or more of the four species studied, but the composition of each species blend was unique (Figure 2).

DISCUSSION

Chemical Composition of Fig Floral Blend. Composition of the blend of *F. fulva* female was similar to that found in our previous study (Grison et al., 1999). In that study, we only identified linalool, β-ocimene, α-copaene, β-caryophyllene, α-humulene, and β-selinene. The first five compounds were present in the sample studied here, and we identified several additional compounds. This is probably due to differences in concentrations: the previous samples were less concentrated, and the sensitivity of the GC-MS did not allow us to identify some minor compounds. Odors from *F. condensa* (male) and *F. microcarpa* have different compositions from those found in our previous work, because the compounds identified then (Grison et al., 1999) were not present in the scents collected in this study. The previous collections also were done in Brunei, but during the drought, due to El Niño in May 1998. The situation for fig trees at that time was unusual, because pollinator populations were absent from the site when the study took place (Harrison, 2000). They came back only several months later [pollinators of *F. condensa* were first seen in the beginning of November 1999 (S. Patiño, personal communication)]. In the absence of pollination, figs seemed to stay receptive for two to three weeks, until aborted.

During the first study, collections were performed on morphologically receptive figs, but since no biological tests could be performed, it was not possible to determine if these figs were truly still receptive. Moreover, volatile emissions during this period may have been affected by the drought. Leaf fall frequently occurred, and even some figs pollinated before the drought aborted (E. Jousselin, personal communication). These data suggest that an environmental stress might have an impact on fig blend composition, and we cannot exclude the hypothesis of a high intraspecific chemical variability. This is highly improbable, however, because in this case, it would be difficult to understand how pollinators could recognize their partner. Some results on *F. carica*, the edible fig, indicate variability among trees in the proportions of the compounds present in the blend but strong similarities in the identities of the compounds of the blends (Grison-Pigé et al., 2001). Additional experiments in years when pollinators are present (such as in the present study) should help to answer the question of the influence of the drought

on fig scent in 1998 and explore the intraspecific variability of the scent of these tropical fig blends.

Specific Stimulation Induced by Floral Blend. The tests of contact stimulation with the associated pollinators were generally significant, even if the length of time that insects stayed on the marbles was variable. Pollinators of *F. fulva*, *F. microcarpa*, and *F. condensa* were clearly stimulated by the odor released by receptive figs of their mutualist. The substances we examined led to an arrestment response of the wasp: the insect stopped, antennated, and flew away after a while. Tests with *F. fulva* male scent showed a nonsignificant trend in the same direction. Solution of this scent was not abundant enough to allow more repetitions. Since compounds in the solution are volatile and since we showed in this study that they have an effect on their associated pollinating wasps, they might also be involved in long-distance attraction of insects. In this study, we did not attempt to distinguish between contact chemoreception and olfaction. Tests of attraction of pollinators at a distance, with a four-branched olfactometer (Vet et al., 1983) or using odor traps, are in progress to examine this. Previous studies with the *F. carica–B. psenes* system (Hossaert-McKey et al., 1994; Gibernau et al., 1998) showed that the same blend was responsible for the attraction of the pollinators of *F. carica* at long distance and for their stimulation once on the fig surface. Gibernau (1997) showed that the same three compounds are responsible for the insect reaction, at both steps. Van Noort et al. (1989) showed that pollinators of *F. burtt-davyi* were attracted to receptive figs by volatile chemical signals, as did Ware and Compton (1994) with pollinators of *F. thonningii*. Thus, data on several species from subgenera *Urostigma* (sections *Americana*, two species, *Conosycea*, one species and *Galoglychia*, two species), *Ficus* (section *Ficus*, subsections *Ficus*, one species, and *Eryosycea*, one species) and *Sycomorus* (one species) show that wasps are receptive to volatile chemical signals released by receptive figs of the appropriate species, at one or both of the two steps of pollinator arrival: distance attraction and stimulation once on the fig. Hence, in three of the four subgenera of genus *Ficus* [morphology (Berg and Wiebes, 1992), molecular phylogeny (Weiblen, 2000)], chemical stimulation of the specific pollinators, sometimes at long distance, has been demonstrated. This suggests that the importance of chemical signals as mediators of the encounter of the two partners is characteristic of the genus.

Tests performed with scent from figs of *F. deltoidea* were not significant. This could be due to the existence of different varieties that were identified in our sample of the species. Fig size and shape differed among individuals. These varieties could constitute good species. Indeed, our sample seemed to come from two different varieties (*F. deltoidea* var. *arenaria* and var. *borneensis*) that are pollinated by different wasp species (F. Kjellberg and J.Y. Rasplus, personal communication). A similar situation was observed by Bergström et al. (1992) on lady's slipper (*Cypripedium calceolus*, Orchidaceae), where three intraspecific taxa exist.

They are pollinated by different species of bees and emit different odors. However, these taxa are geographically distinct, whereas in the case of *F. deltoidea* the different morphs can be found at the same site. Thus, in the case of *F. deltoidea*, the two morphs are probably already reproductively separated, whereas in the case of *Cypripedium calceolus*, this speciation is presumably in process. In the genus *Narcissus* (Amaryllidaceae), Dobson et al. (1997) observed that one species could include different chemotypes, in relation with different major pollinators. Unfortunately, odors of the different morphs of *F. deltoidea* were not analyzed separately, because of the necessity to combine samples to have concentrated extracts for GC-MS analyses. Comparisons of the scents would be interesting, in order to test the hypothesis of the existence of chemotypes in this species or of sibling species in which differential pollinator attraction plays a role in reproductive isolation.

Specificity of Interaction. Crossed bioassays were performed with odors shown to be active on the specific pollinator (Figure 1). Odors of the species studied often shared several compounds (Figure 2), but in five of the six cross-tests performed, pollinators were not stimulated by the floral blend of a species other than their associated one. This suggests that several compounds of the blend contribute to the signal. Two nonexclusive hypotheses can be advanced. The first one is that not only are the identities of the molecules important, but also that their ratios must be part of the scent specificity. This was previously shown by Nordlander et al. (1986) on weevil attraction by pine volatiles and Roseland et al. (1992) on weevil attraction to the sunflower and was discussed by Visser (1986). The second one is that some compounds may be below the threshold of the GC-MS detection but may be responsible for wasp stimulation. This would reveal a keen coadaptation between the partners of the interaction (Guerin and Städler, 1982; Baur and Feeny, 1995). Which one of these hypotheses applies here has not yet been determined, and further bioassays are needed. This first experiment confirms the role of the chemical signal, which allows the specificity of the encounter, thus maintaining the association. To determine which step(s) (distance attraction or contact stimulation of the insect) guarantees in natura the specificity of the encounter, other types of bioassays are needed, for example, with a four-branched olfactometer (Vet et al., 1983). The role of fig scent as specific attractant of pollinators has been suggested by Van Noort et al. (1989) for *F. burtt-davyi* and by Ware and Compton (1994) for *F. burtt-davyi* and *F. thonningii*.

Odors are considered to contribute to reproductive isolating mechanisms in the genus *Catasetum* (Orchidaceae), where species having a specific odor also have a specific pollinator (Hills et al., 1972). In *Cimicifuga simplex*, evolution of the composition of the scent can be considered as an adaptation to pollination by a new guild of pollinators under variable environmental conditions (Pellmyr, 1986; Groth et al., 1987). In such a system, pollination morphs would probably evolve to distinct species. For one of the six tests (odor from *F. fulva* and pollinators of *F. microcarpa*), a significant difference was observed between the times the insects

stayed on the control and the sample-treated marbles (Figure 1). Pollinators of *F. microcarpa*, thus, seem to be at least somewhat stimulated by odor from *F. fulva* figs. This could be an effect of the common compounds of the scents of the two species: they share 8 of the 13 and 15 compounds (of abundance $>1\%$) constituting their respective odors. Other tests on receptive figs are necessary to determine whether this stimulation is strong enough to induce the insects to enter the fig and lay eggs inside. These two species belong to different subgenera and do not have the same natural habitats, but they can be found at short distances from each other (a few hundred meters, for example, in the city). At the moment, our observations in the field do not suggest that this breakdown of specificity really occurs. Breakdowns of specificity, however, have been observed for several *Ficus* species (Compton, 1990; Gibernau, 1997). Odors were not analyzed in studies of these breakdowns, but it would be interesting to compare them for a better understanding of the limits of their role as isolating mechanisms. These cross-tests were performed with species belonging to different subgenera, and it would be interesting to investigate the specificity of the stimulation by chemical signals for pollinators of more or less closely related species.

Combining bioassays and chemical analyses, we were able to show, for three tropical *Ficus* species, the role of the chemical signal as pollinator stimulant and the specificity of the stimulation. Chemical analyses strongly suggest the importance of compound ratios in the blend for recognition by specific pollinating wasp.

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ANTIPREDATOR DEFENSE OF BIOLOGICAL CONTROL AGENT *Oxyops vitiosa* IS MEDIATED BY PLANT VOLATILES SEQUESTERED FROM THE HOST PLANT *Melaleuca quinquenervia*

G. S. WHEELER,[1,*] L. M. MASSEY,[2] and I. A. SOUTHWELL[3]

[1]*USDA/ARS Invasive Plant Research Laboratory*
and University of Florida
3205 College Ave
Ft. Lauderdale, Florida 33314

[2]*Department of Occupational Therapy*
Nova Southeastern University
Ft. Lauderdale, Florida 33314

[3]*New South Wales Agriculture*
Wollongbar Agricultural Institute
Wollongbar, NSW, 2477, Australia

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Abstract—The weevil *Oxyops vitiosa* is an Australian species imported to Florida, USA, for the biological control of the invasive weed species *Melaleuca quinquenervia*. Larvae of this species feed on leaves of their host and produce a shiny orange secretion that covers the integument. When this secretion is applied at physiological concentrations to dog food bait, fire ant consumption and visitation are significantly reduced. Gas chromatographic analysis indicates that the larval secretion qualitatively and quantitatively resembles the terpenoid composition of the host foliage. When the combination of 10 major terpenoids from the *O. vitiosa* secretion was applied to dog food bait, fire ant consumption and visitation were reduced. When these 10 terpenoids were tested individually, the sesquiterpene viridiflorol was the most active component in decreasing fire ant consumption. Fire ant visitation was initially (15 min after initiation of the study) decreased for dog food bait treated with viridiflorol and the monoterpenes 1,8-cineole and α-terpineol. Fire ants continued to avoid the bait treated with viridiflorol at 18 μg/mg dog food for up to 6 hr after the initiation of the experiment. Moreover, ants avoided bait treated with 1.8 μg/mg for up to 3 hr. The concentrations of viridiflorol, 1,8-cineole, and α-terpineol in larval washes were about twice that of the host foliage, suggesting that the larvae sequester these plant-derived compounds for defense against generalist predators.

*To whom correspondence should be addressed. E-mail: wheelerg@eemail.com

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Key Words—Antipredator defense, biological control of weeds, Curculionidae, generalist predator, *Melaleuca quinquenervia*, Myrtaceae, *Oxyops vitiosa*, plant defensive compounds, red imported fire ant, sequestration, *Solenopsis invicta*, terpenoids, viridiflorol.

INTRODUCTION

The Australian weevil, *Oxyops vitiosa*, Pascoe (Coleoptera: Curculionidae) was introduced in south Florida in 1997 for the biological control of the invasive weed *Melaleuca quinquenervia* (Cav.) S. T. Blake (Myrtaceae) (Center et al., 2000). This weevil species has since been established throughout the infested area of Florida. Larvae feed on young leaves and have a characteristic odor that resembles that of the foliage of their host tree. The bright orange larvae are diurnally active and feed exposed during all instars on the foliage, then drop to the ground, where they form a protective pupal cell in the soil (Purcell and Balciunas, 1994). The cuticle of the larvae is completely covered with a shiny, oily substance that may function in defense against generalist predators (Montgomery and Wheeler, 2000). However, the chemical nature of this defense has yet to be determined. Although few weevil species have been reported to produce defensive secretions (Pavis et al., 1992), similar integumental slime found on the sluglike sawfly larvae of *Caliroa cerasi* reduced the incidence of predation by generalist ants (Eisner, 1994).

The red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae) constitutes one of the most invasive invertebrate predators in the southeastern United States, invading forests, pastures, croplands, and natural areas (Lofgren et al., 1975). This ant is among the most common invertebrate predators in the southeastern United States. (Elvin et al., 1983; Kharboutli and Mack, 1991). Generalist predators, including ants, have prevented the establishment or reduced the effectiveness of many weed biological control agents (Goeden and Louda, 1976), and this ant species, in particular, has been directly implicated in reducing the efficacy of *Tyta luctuosa*, a biological control agent of field bindweed (Ciomperlik et al., 1992).

Biological control efforts may benefit by recruiting agents that sequester plant defensive compounds that impart protection from generalist predators. Sequestration of plant defenses may be more common than appreciated by biological control practitioners, as this phenomenon is common among herbivores with narrow host ranges (Bowers, 1990). Several weed biological control agents are known to sequester plant defensive compounds, namely the pyrrolizidine alkaloids of the *Senecio jacobaea–Tyria jacobaeae* association (Aplin et al., 1968), the quinolizidine alkaloids of the Scotch broom–aphid association (Wink et al., 1982), and the bisanthraquinone hypericin obtained by the *Chrysolina* beetles from their host plant *Hypericum* (Rees, 1969). The purpose of this study was to determine the nature of the defensive secretions produced by the larvae of *O. vitiosa* toward the generalist predator *S. invicta*.

METHODS AND MATERIALS

Insects. Larvae of *O. vitiosa* were collected ($N = 20$) from a field site infested with *M. quinquenervia* in Ft. Lauderdale, Broward County, Florida. All larvae were weighed alive prior to analysis. Solvent washes were conducted on fourth instars by dipping each in 1 or 2 ml of chloroform ($CHCl_3$) for 15 sec. The $CHCl_3$ larval wash was filtered through glass wool and dried over sodium sulfate (Na_2SO_4) prior to analysis by gas chromatography.

Red imported fire ants were collected at the University of Florida, Ft. Lauderdale Research and Education Center, as described previously (Montgomery and Wheeler, 2000). Colonies, consisting of brood, eggs, and workers, were established in plastic boxes (20 × 12 cm) and provided with deionized water in a glass tube (45 × 4 mm) plugged with cotton. Ants were fed kibbled dog food (Pedigree Prime) freely for two to five days prior to each test. The food was removed 24–36 hr prior to each test. Newly collected ant colonies were used for each test.

Plants. Leaves were collected from the same location as the larvae described above. Tip leaves were clipped from young trees and brought to the laboratory where they were frozen (−10°C) for further processing. The leaf components were extracted by a modified microwave technique (Southwell et al., 1995; Degen and Stadler, 1998; Gomez et al., 1999). Fresh leaf samples (75–150 mg fresh weight) were immersed in 1 ml EtOH (95%) and irradiated with microwaves (750 W) for 60 sec. An aliquot (500 μl) of the EtOH extract was mixed with deionized water (500 μl), followed by 500 μl of $CHCl_3$. The sample was vortexed for 1 min and then centrifuged for 10 min at 10,000 rpm. An aliquot (200 μl) of the $CHCl_3$ layer was removed and dried over Na_2SO_4. Internal standards were added as 10 ng/μl each of *n*-tridecane and *n*-eicosane in 10 μl $CHCl_3$ to the extract prior to analysis by gas chromatography.

Chemicals. Terpenoids were purchased from commercial sources, except where mentioned, and were of the highest purity available. The list included the primary compounds reported by Brophy et al. (1989) and Ramanoelina et al. (1994). These included (+)-α-pinene (Chemical Purity = 98%), (−)-β-pinene (99%), α-terpinene (98%), (−)-limonene (92%), 1,8-cineole (100%), γ-terpinene (98%), terpinen 4-ol (97%), α-terpineol (98%), and *trans*-nerolidol (95%). Viridiflorol (86%) was extracted from *M. quinquenervia* foliage by one of us (I.A.S.).

Gas Chromatography. Samples were analyzed with either a Hewlett-Packard model 6890 or 5890 gas chromatograph. Data collection, storage, and analysis were conducted with the ChromPerfect data system. Helium at a linear flow rate of 37 cm/sec was used as a carrier gas. All samples were analyzed on three fused silica capillary columns [HP-5 (Hewlett Packard Company, Wilmington, Delaware), DB-17MS and a DB-WAXetr (J & W Scientific, Folsom, California); all 30 m × 0.32 mm ID, 0.25-μm-thick film]. Injector temperature was 200°C and FID temperature was 250°C. The oven temperature was held at 50°C for 2 min then increased

at 8°C/min to 250°C, where it was held for 5 min. Compounds were quantified relative to the peak areas of the internal standard and values were adjusted by calculation of their response factors relative to *n*-tridecane (Debbrecht, 1985).

Compound identities were confirmed by GC-MS using a Hewlett Packard 6890 instrument fitted with a HP-5MS (30.3 m × 0.25 mm, 0.25-μm film thickness) FSOT column with helium (36 cm/sec) as a carrier gas, injector port (split 1:50) at 250°C, mass selective detector (HP 5973) at 250°C (source) and 150°C (quad) with transfer line 280°C and ion source filament voltage of 70 eV. Component identification was made on the basis of mass spectral fragmentation, retention index with *n*-paraffins, and comparison with authentic constituents and mass spectral and retention matching with commercial (NIST, Wiley, and Adams) libraries.

Bioassay of Defensive Secretions. The material washed from the *O. vitiosa* larvae or select commercial terpenoids was dissolved in 50 μl of $CHCl_3$ and applied to kibbled dog food cut into 50-mg pieces. The solvent was allowed to dry for 0.5 hr in a fume hood under ambient conditions. The treated dog food bait was stored for 12–15 hr at 4°C. Each test consisted of offering the ants a choice between a solvent ($CHCl_3$)-treated control and either a crude larval wash or a select commercial terpenoid formulated individually or in a mixture. To determine the percentage loss of terpenoids during the experiment, treated dog food samples were $CHCl_3$-extracted before and after a 6-hr test and analyzed by GC. By comparing peak areas the concentration (mean ± SE) of each terpenoid was found to decrease during the experiment from 53.9% ± 2.5% for viridiflorol to 93.7% ± 0.9% for α-pinene.

Four choice tests were conducted, all under ambient conditions between 9 AM and 3 PM. For all tests, ant consumption was determined by comparing the dog food weight before and after each 6-hr test. Additionally, the number of ants visiting each dog food bait was counted at regular intervals. In study 1, choice tests were conducted between a $CHCl_3$-treated control and the crude *O. vitiosa* larval wash [1-larval equivalent (LE)] applied to a piece of dog food ($N = 10$). In study 2, choice tests were conducted between a $CHCl_3$-treated control and a mixture of the 10 major terpenoids found from GC analysis of the larval washes ($N = 6$). These were formulated to include the range of concentrations of each compound found in the crude larval washes (Table 1). Additional treatments consisted of 10-fold and 100-fold dilutions of these mixtures. One major *M. quinquenervia* terpenoid, β-caryophyllene, was not identified during this phase of the research and, thus, its repellent activity was not determined. In study 3, choice tests were conducted between a $CHCl_3$-treated control and the same 10 terpenoids tested individually at the highest concentrations tested in study 2 ($N = 4$). In study 4, choice tests were conducted between a $CHCl_3$-treated control and three different viridiflorol concentrations using the same concentrations as in study 2 ($N = 6$).

Statistical Analysis. All data, terpenoid extraction yields, *S. invicta* consumption, and the number of fire ants visiting each dog food bait, were analyzed with

TABLE 1. CONCENTRATION OF 10 TERPENOID COMPONENTS APPLIED TO KIBBLED DOG FOOD AND FED TO RED IMPORTED FIRE ANT WORKERS[a]

Compound	Concentration (μg/mg dog food)
1,8-Cineole	22.00
Viridiflorol	18.00
trans-Nerolidol	5.02
α-Terpineol	5.47
α-Pinene	5.06
Limonene	3.09
β-Pinene	1.70
Terpinen 4-ol	1.80
γ-Terpinene	1.67
α-Terpinene	1.43

[a] Each piece of dog food (50 mg) was treated with 1-fold (high), 10-fold (intermediate) or 100-fold (low) dilutions of one larval equivalent of these 10 components ($N = 6$).

SAS (SAS Institute, 1990). The terpenoid yields of the leaf extracts and the larval washes were compared with an ANOVA for each terpenoid analyzed. The consumption and the fire ant activity data were analyzed with a Kruskal-Wallis test.

RESULTS

Chemical Analyses

Considerable quantitative and qualitative similarity was found in the chemical constituents from leaf extracts and larval washes (Table 2; Figure 1). However, some of the terpenoids analyzed (e.g., viridiflorol, 1,8-cineole, α-terpineol, limonene, terpinen 4-ol) were present in greater concentrations in the larval washes than in the leaf extracts. This suggests that as the *O. vitiosa* larvae consume *M. quinquenervia* foliage, they absorb and deposit the foliar terpenoids on their cuticle. The larval fresh weight averaged ($\pm$SE) 101.9 ($\pm$3.0) mg and the amount of material removed by washing each larva averaged 7.9 ($\pm$0.5) mg.

Bioassay of Larval Secretions

Study 1—Larval Washes. Fire ant consumption and visitation were significantly greater on the control dog food bait (treated with $CHCl_3$) compared with that treated with the *O. vitiosa* larval wash (Table 3). During the 6-hr test, the fire

TABLE 2. COMPARISON OF MEAN (±SE) CONCENTRATIONS OF 10 TERPENOID COMPONENTS FROM $CHCl_3$-EXTRACTED *M. quinquenervia* LEAVES ($N = 4$) AND $CHCl_3$-WASHED *O. vitiosa* LARVAE ($N = 10$)[a]

	Leaf extract		Larval wash				
Compound	μg/mg	SE	μg/mg	SE	*F*	*df*	*P*
Viridiflorol	6.06	0.86	11.85	1.25	5.59	1,13	0.034
1-8 Cineole	3.15	0.41	6.73	0.76	7.67	1,13	0.016
trans-Nerolidol	nd		nd				
α-Terpineol	0.89	0.12	2.23	0.22	11.96	1,13	0.004
α-Pinene	1.47	0.35	1.44	0.18	0.01	1,13	>0.900
β-Pinene	0.44	0.08	0.45	0.04	0.01	1,13	>0.900
Limonene	1.13	0.16	0.66	0.08	5.86	1,13	0.031
γ-Terpinene	0.08	0.01	0.09	0.01	1.13	1,13	>0.300
Terpinen 4-ol	0.07	0.01	0.15	0.01	24.33	1,13	<0.001
α-Terpinene	0.01	0.00	0.03	0.01	4.02	1,8	0.080

[a] Results of ANOVA are presented for comparisons between the leaf extract and larval washes for each compound. Values estimated by gas chromatography and expressed as μg/mg of fresh leaf or larval weight.
nd: not detected.

TABLE 3. CONSUMPTION AND VISITATION BY RED IMPORTED FIRE ANTS OF DOG FOOD TREATED WITH $CHCl_3$ (CONTROL) OR *O. vitiosa* LARVAL WASHES (6 HR TEST)[a]

			Number fire ants visiting bait			
	Consumption (mg)		3 hr		6 hr	
Colony	Control	Treated	Control	Treated	Control	Treated
1	4.4	1.5	10	0	9	0
2	2.8	0.7	7	0	11	0
3	11.2	1.8	10	0	12	0
4	4.3	2.3	8	0	7	0
5	40.0	0.7	30	0	30	0
6	3.7	0.7	15	0	10	0
7	9.0	0	10	0	6	0
8	3.0	0	17	0	11	0
9	4.7	0	12	0	7	0
10	3.9	0	13	0	9	0
Mean	8.7	0.8	13.2	0	11.2	0
SE	3.6	0.3	21	0	2.2	0
	$\chi_1^2 = 14.438$; $P < 0.001$		$\chi_1^2 = 16.365$; $P < 0.001$		$\chi_1^2 = 16.351$; $P < 0.001$	

[a] Each piece of dog food (50 mg) was treated with one larval equivalent.

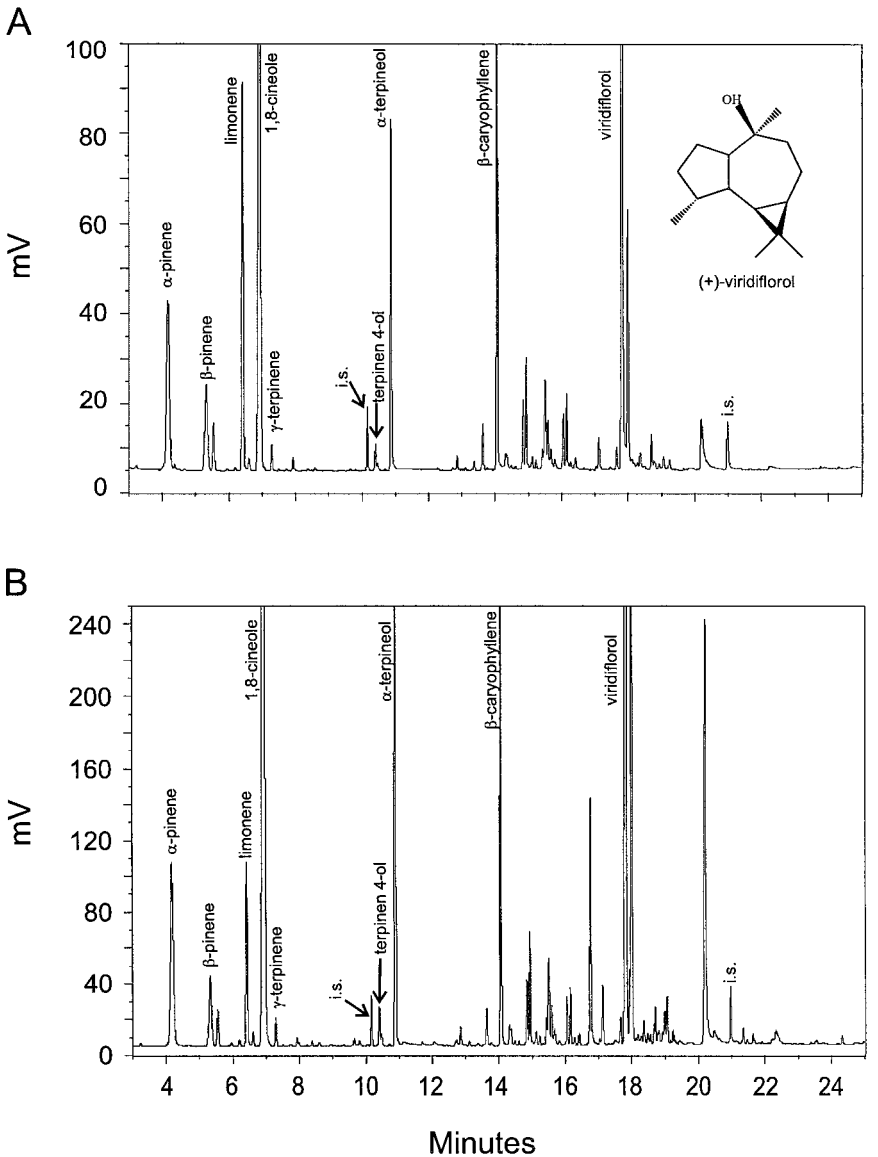


FIG. 1. Representative gas chromatograms of *M. quinquenervia* leaves (A) and *O. vitiosa* larval washes (B). Leaves were digested in 95% EtOH by 60-sec microwave irradiation. Larval washes consisted of dipping fourth-instar larvae in $CHCl_3$ for 15 sec. Internal standards *n*-tridecane and *n*-eicosane (10 ng/μl of each) were added to each sample.

ants consumed an average of 8.7 (±3.6) mg of dog food in the controls compared with 0.8 (±0.3) mg of dog food in the larval-wash treatment. Additionally, fire ants frequently visited the control dog food at both 3 and 6 hr after initiation of the experiment, whereas they were never observed visiting the larval wash-treated dog food (Table 3).

Study 2—Mixture of Terpenoids. Fire ant consumption and visitation of dog food were significantly lower following treatment with the mixture of the 10 terpenoids (about 1 LE) than with the solvent-treated controls. In the high ($\chi_1^2 = 8.932$; $P = 0.003$) treatment of the terpenoid mixture, consumption was significantly reduced compared with that of the solvent-treated dog food (Figure 2). Furthermore, fire ant visitation was reduced (Figure 3) during the 15-min observation period in the high ($\chi_1^2 = 9.466$; $P = 0.002$), intermediate ($\chi_1^2 = 8.425$; $P = 0.004$), and low ($\chi_1^2 = 8.366$; $P = 0.004$) treatments of the terpenoid mixture

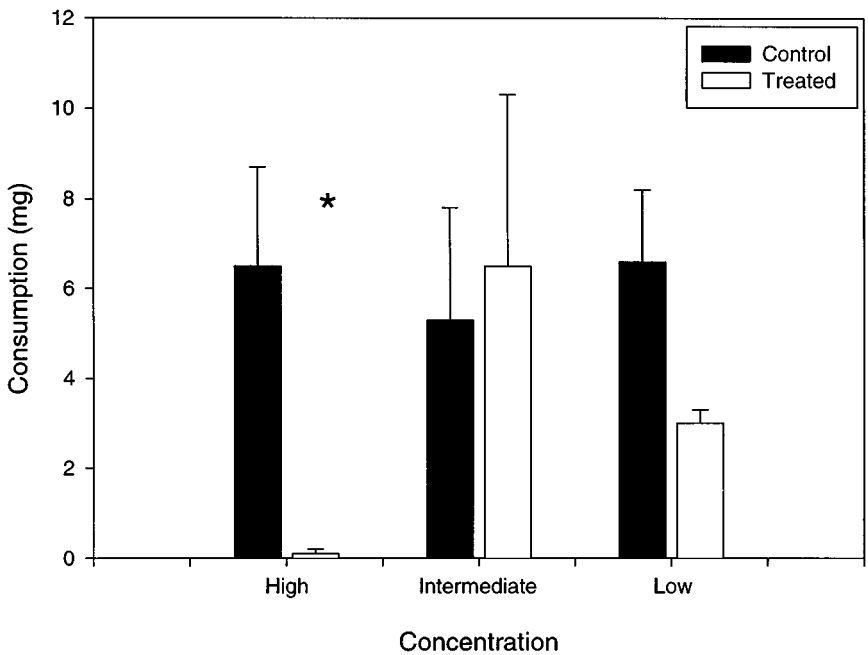


FIG. 2. Mean consumption (±SE) by *S. invicta* workers offered a choice between a dog food bait treated with $CHCl_3$ (control) and a mixture of the 10 major terpenoids occurring in $CHCl_3$ washes of *O. vitiosa* larvae. The high concentration approximated the amount washed from one larva (Table 1), whereas the intermediate and the low levels were 10-fold and 100-fold dilutions, respectively. Asterisk indicates significant differences between the control and treated means.

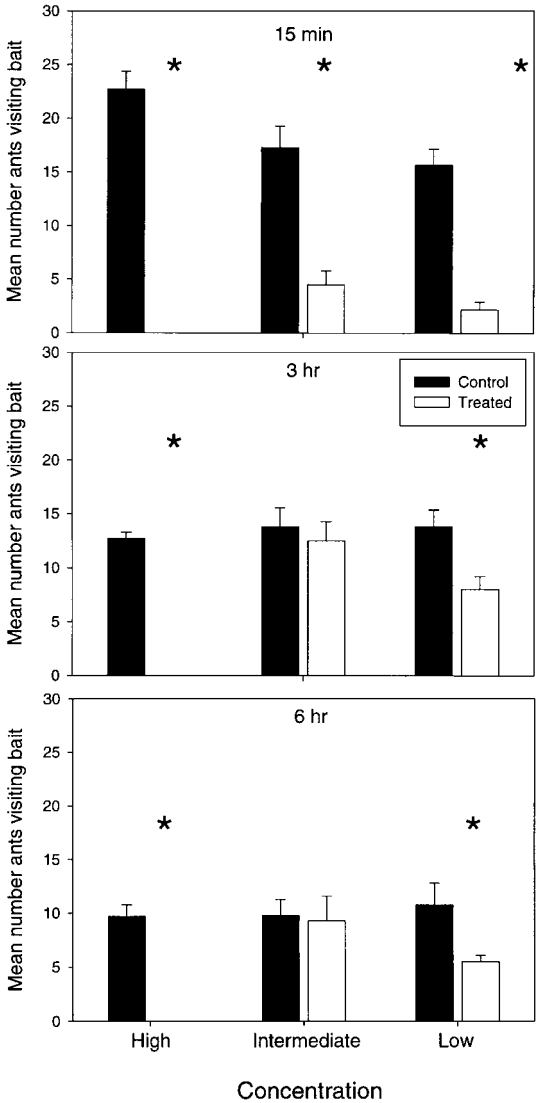


FIG. 3. Mean number (±SE) of *S. invicta* workers visiting dog food bait treated with $CHCl_3$ (control) and a mixture of the 10 major terpenoids occurring in $CHCl_3$ washes of *O. vitiosa* larvae. The high concentration approximated the amount washed from one larva (Table 1), whereas the intermediate and the low levels were 10-fold and 100-fold dilutions, respectively. The number of ants contacting each dog food bait was counted at 15 min, 3 hr, and 6 hr after the beginning of the experiment. Asterisks indicate significant differences between the control and treated means.

compared with the solvent-treated controls. Significant differences were also found 3 hr after the study began in the high ($\chi_1^2 = 9.542$; $P = 0.002$) and the low ($\chi_1^2 = 5.448$; $P = 0.020$) concentrations. Finally, fewer fire ants were observed visiting the terpenoid mixture-treated dog food at 6 hr after the study began on the high ($\chi_1^2 = 9.466$; $P = 0.002$) and the low ($\chi_1^2 = 6.226$; $P = 0.012$) concentrations.

Study 3—Individual Terpenoids. When each terpenoid was applied individually to the dog food, the only treatments that reduced fire ant consumption of bait were α-terpineol (Figure 4; 5.47 μg/mg; $\chi_1^2 = 5.333$; $P = 0.021$) and viridiflorol (18.0 μg/mg; $\chi_1^2 = 5.398$; $P = 0.020$). Fire ant visitation was reduced after 15 min (Figure 5) in those treated with 1,8-cineole (22.0 μg/mg; $\chi_1^2 = 3.607$; $P = 0.058$), α-terpineol ($\chi_1^2 = 4.083$; $P = 0.043$) and viridiflorol ($\chi_1^2 = 5.463$; $P = 0.019$).

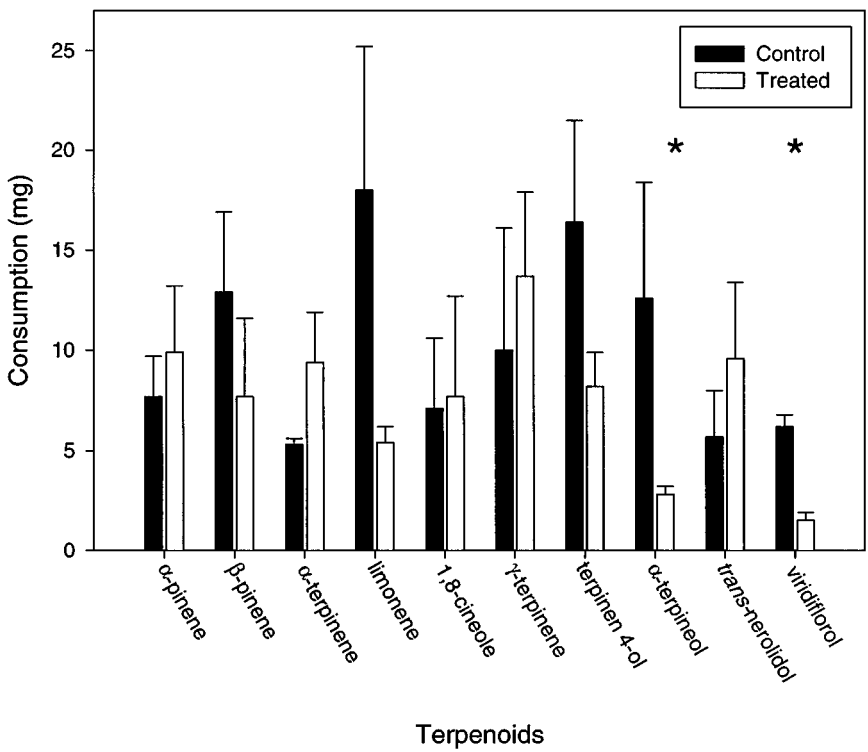


FIG. 4. Mean consumption (±SE) by *S. invicta* workers offered a choice between a dog food bait treated with $CHCl_3$ (control) and each of the 10 major terpenoids occurring in $CHCl_3$ washes of *O. vitiosa* larvae. Asterisks indicate significant difference between the control and treated means.

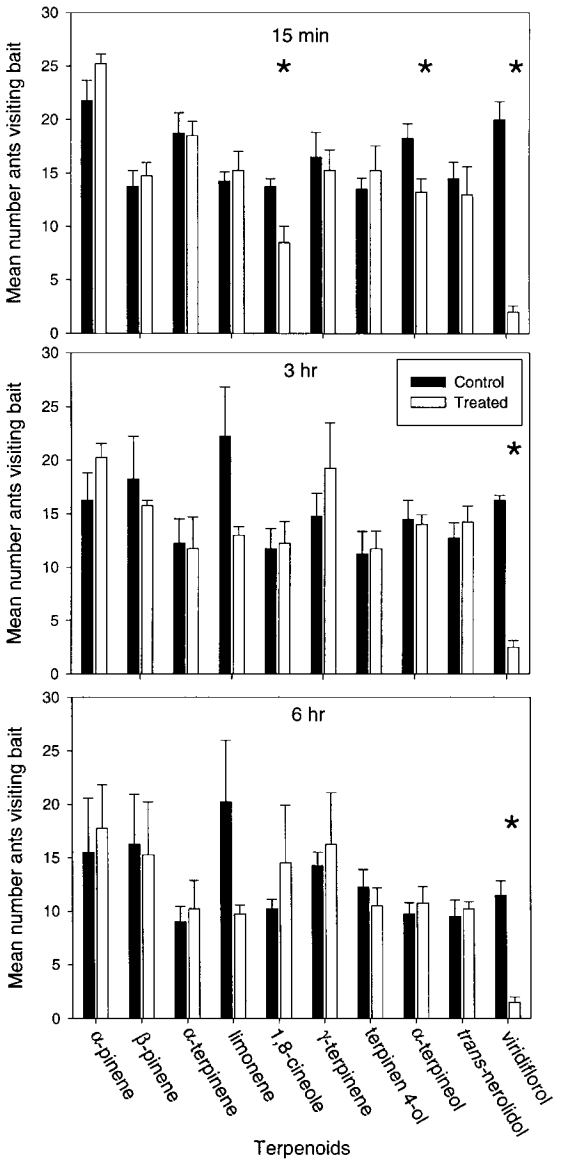


FIG. 5. Mean number (±SE) of *S. invicta* workers visiting dog food bait treated with $CHCl_3$ (control) and each of the 10 major terpenoids occurring in washes of *O. vitiosa* larvae. The number of ants contacting each dog food bait was counted at 15 min, 3 hr, and 6 hr after beginning the experiment. Asterisks indicate significant differences between the control and treated means.

After 3 hr ($\chi_1^2 = 5.398$; $P = 0.020$) and 6 hr ($\chi_1^2 = 5.600$; $P = 0.018$) fire ant visitations were reduced only on the viridiflorol-treated dog food bait.

Study 4—Viridiflorol. Viridiflorol tested alone was active over a range of concentrations at decreasing fire ant consumption and repelling ants. Compared with the solvent-treated controls, consumption was significantly reduced only by viridiflorol at the highest concentration ($\chi_1^2 = 4.395$; $P = 0.036$) tested (Figure 6). However, fire ant visitation to viridiflorol-treated bait was reduced at the highest ($\chi_1^2 = 9.466$; $P = 0.002$) and intermediate ($\chi_1^2 = 9.103$; $P = 0.003$) concentrations 15 min after the beginning of the study (Figure 7). This activity continued for 3 hr after the beginning of the study at the highest ($\chi_1^2 = 9.466$; $P = 0.002$) and intermediate ($\chi_1^2 = 3.480$; $P = 0.062$) viridiflorol concentrations. After 6 hr, a difference in activity was only found at the highest viridiflorol concentration ($\chi_1^2 = 7.174$; $P = 0.007$).

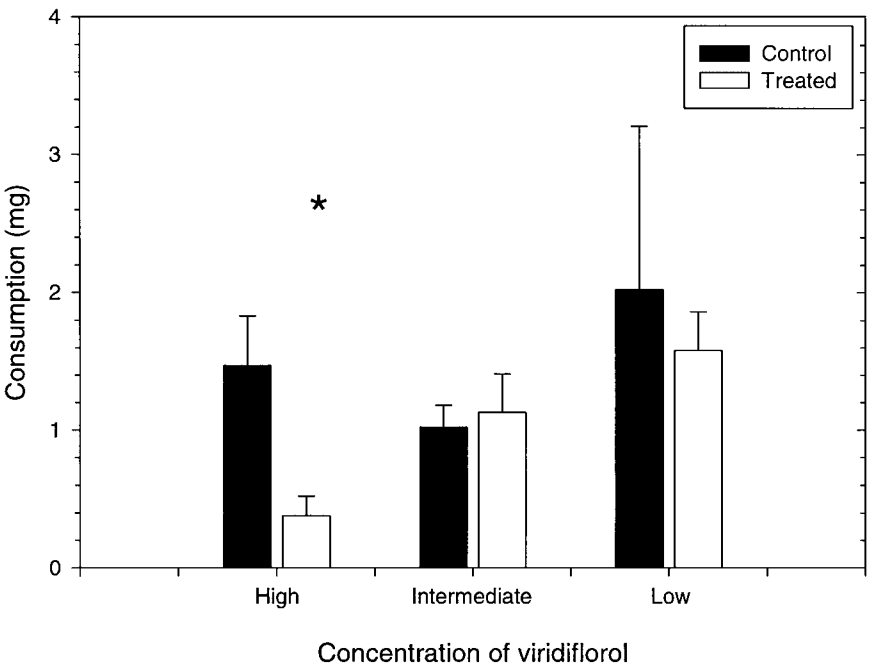


FIG. 6. Mean consumption (±SE) by *S. invicta* workers offered a choice between a dog food bait treated with $CHCl_3$ (control) and viridiflorol at high (18 μg/mg dog food bait), intermediate (1.8 μg/mg dog food bait), and low (0.18 μg/mg dog food bait) concentrations. Asterisk indicates significant difference between the control and treated means.

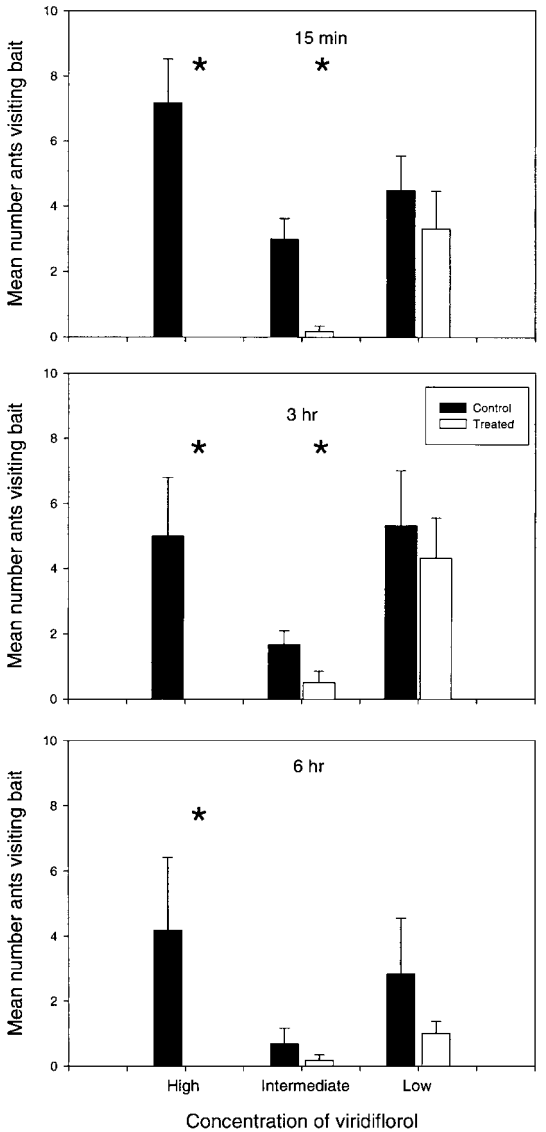


FIG. 7. Mean number (±SE) of *S. invicta* workers visiting dog food bait treated with $CHCl_3$ (control) and viridiflorol at high (18 μg/mg dog food bait), intermediate (1.8 μg/mg dog food bait) and low (0.18 μg/mg dog food bait) concentrations. The number of ants contacting each dog food bait was counted at 15 min, 3 hr, and 6 hr after beginning the experiment. Asterisks indicate significant differences between the control and treated means.

DISCUSSION

These results demonstrate the repellent nature of the larval secretions of the *M. quinquenervia* biological control agent *O. vitiosa* toward the red imported fire ant *S. invicta*. They indicate repellent activity from the crude larval washes, mixtures of the 10 major terpenoids contained in the larval washes, and individual compounds from this mixture. In all experiments, fire ant consumption and visitation to the treated baits significantly decreased compared with the solvent-treated controls. The repellency of the most active component, viridiflorol, was demonstrated throughout the duration of the experimental observations (6 hr). Additionally, 1,8-cineole and α-terpineol were repellent during the initial observation (15 min). Possibly limonene was also repellent, although variable, and thus was not statistically significant in these analyses.

In the larval secretions, the three most active compounds, viridiflorol, 1,8-cineole, and α-terpineol, were recovered at about twice the concentration extracted from the host foliage, suggesting that the larvae sequester these plant-derived compounds. In fact, nearly all of the 10 compounds investigated were in greater concentration (on a fresh mass basis) washed from the larvae compared with those measured in the leaf extracts. The most active ant repellents increased the most from the plant to the larvae, namely viridiflorol (2.0-fold), 1,8-cineole (2.1-fold), and α-terpineol (2.5-fold). The proportion of ingested terpenoids retained by larvae on the cuticle can be calculated and expressed as the conversion efficiency for each terpenoid (ECI_t), analogous to calculation of the food conversion efficiency (ECI_f) (Slansky and Scriber, 1985). The ECI_t values were estimated by: [larval mass (mean = 101.9 mg) $\times$ concentration of the terpenoid (μg/mg) washed from the larvae (from Table 2)]/[food consumed (mg) $\times$ concentration of the terpenoid (μg/mg) in the leaves (from Table 2)]. We used consumption (400 mg fresh mass) and food conversion efficiency (ECI_f; 7.3% fresh mass) results from a similar study of *O. vitiosa* larvae fed leaves of *M. quinquenervia* (Wheeler, unpublished data). To determine if select terpenoids were actively sequestered and deposited on the larvae, the conversion efficiency of each active terpenoid washed from the cuticle (ECI_t) can be compared with the conversion efficiency of the food (ECI_f). The results of these calculations generally suggest that more than half the quantity consumed of the most repellent terpenoids was retained and deposited on the larval cuticle. For example, the ECI_t values all exceeded 46% for viridiflorol (49.8%), 1,8-cineole (54.5%), and α-terpineol (63.9%). Moreover, with the exception of α-terpinene and terpinen 4-ol, which were present in barely detectable levels, the conversion efficiencies of the less-repellent terpenoids were considerably lower ($\leq$30%). These results are consistent with the interpretation that these terpenoids were actively sequestered, as these values were considerably greater than the conversion efficiency value for food ($ECI_f = 7.3\%$). A similar analysis was conducted, using the approximate digestibility (AD) of the

ingested terpenoid 1,8-cineole by a chrysomelid eucalypt-feeder, *Paropsis atomaria* (Olivier) (Ohmart and Larsson, 1989). Our calculations also suggest that sequestration was selective as the ECI_t values were greatest for the most repellent compounds. Similar selectivity was found with the larvae of other species where the most emetic iridoid glycoside, catalpol, was selectively sequestered compared with the less-emetic aucubin (Bowers and Collinge, 1992; Bowers et al., 1993). However, our calculations need to be confirmed with a more direct determination of the terpenoid budgets. Finally, our intention was to test the terpenoids at physiological concentrations. Thus, as the most repellent compounds were the most abundant terpenoids washed from the larvae, they were also tested at higher concentrations. Possibly, α-terpinene and γ-terpinene would be equally active if tested at higher concentrations.

Ant foraging activity was similar in studies 1–3, where typically more than 10 ants was reported on the control bait during each observation and 5 mg of the control bait was consumed. However, a decrease in the number of foraging ants was observed in study 4, resulting in less than 10 foragers reported during all observation periods and less than 2 mg of bait consumed by foragers. Similar variability in foraging activity occurred in a subsequent study (Wheeler et al., unpublished data). Several factors may influence ant activity including degree of satiation. However, the food was always removed from the ant colonies 24–36 hr prior to each test. Possibly seasonal or colony phenology changes influenced foraging activity. However, these factors were minimized as all the studies were conducted during July and August 2000. Moreover, studies were always conducted from 9 AM to 3 PM at ambient temperatures that varied only slightly on the days of the tests, with mean (range) temperatures between 27.3°C (22.8–31.7°C; study 3) and 28.6°C (23.3–33.9°C; study 4). Thus, it is unclear why consumption and foraging activity were lower during study 4.

Terpenoids are well known ant repellents (Shorey et al., 1992) that, when sequestered (e.g., cardenolides, cucurbitacins), may be repellent against many predatory types (Bowers, 1990). Several terpenoids have shown activity against both herbivorous and predaceous ant species. For example, plant foliar terpenoids may either repel (Hubbell et al., 1984; Howard et al., 1989) or be toxic to leaf-cutting ants (Howard et al., 1988). Additionally, insect-produced terpenoids from chrysomelid larvae also repel *S. invicta* (Blum et al., 1978). The plant sesquiterpene farnesol may be applied as a band around citrus tree trunks to exclude Argentine ants *Linepithema humile* (Mayr) for several months (Shorey et al., 1996). Numerous compounds with a range of volatilities have been reported to have ant repellent activity (Shorey et al., 1992); however, to our knowledge, this is the first report of repellent activity at physiological concentrations of the terpenoids viridiflorol, 1,8-cineole, and α-terpineol.

The ecological host range of this biological control agent *O. vitiosa* may be influenced by the availability of foliar compounds that serve as effective repellents

against generalist predators. Larvae may escape from predators on plant species that have the compounds that can be sequestered for antipredator defense (Jeffries and Lawton, 1984). Even though oviposition and larval development may occur on test plants in the laboratory, in field conditions, where natural enemies like fire ants are abundant, host use may be restricted to plant species that provide compounds that can be sequestered and impart defense against generalist predators. Although field observations indicated that this species was only collected on *M. quinquenervia* (Balciunas et al., 1994), quarantine host testing indicated that neonates complete development on the Australian myrtaceous species *Callistemon rigidus* and *C. viminalis* (Balciunas and Buckingham, 1996). Additionally, third instars previously fed *M. quinquenervia* will feed on the North American *Myrica cerifera* (Balciunas and Buckingham, 1996; Wheeler, unpublished data). Additional chemical variation in the *O. vitiosa* host range exists in the different *M. quinquenervia* chemotypes, both of which occur in Australia (Ireland, 1999) and Florida (Dray and Wheeler, unpublished data). The two *M. quinquenervia* chemotypes have distinct terpenoid profiles; one has primarily *trans*-nerolidol and only small concentrations of the primary components found in the present study. Different larval diets influenced the cuticular waxes of *Manduca sexta* when fed different plant species or an artificial diet (Espelie and Bernays, 1989) and these differences influenced the susceptibility of the larvae to ant predation (Cornelius and Bernays, 1995). The protection *O. vitiosa* larvae derive from these plant species and chemotypes and its ecological significance are the subject of an additional series of studies (Wheeler et al., unpublished data).

Predators are well-known antagonists of arthropods used for biological control of weeds (Crawley, 1989; Julien and Griffiths, 1998), and predation is so common that it may be assumed that it likely influences the establishment of all agents (Goeden and Louda, 1976). In general, natural enemies, including predators, parasitoids, and diseases interfere with approximately half of biological control projects (Goeden and Louda, 1976). *Oxyops vitiosa* is apparently well protected from one of the most common invertebrate generalist predators in the southeastern United States (Montgomery and Wheeler, 2000). However, recent observations indicate that another predator, the pentatomid, *Podisus mucronatus* Uhler, with relatively long mouthparts may penetrate the integumental defenses of *O. vitiosa* larvae (Pratt, unpublished data). Different mouthpart sizes have allowed heteropteran predators with long mandibles [e.g., *Nabis americoferus* (Carayon) (Heteroptera: Nabidae) or *Podisus maculiventris* (Say)] (Heteroptera: Pentatomidae) to utilize otherwise well-defended prey compared with predators with shorter mandibles (e.g., coccinelids) (Olmstead and Denno, 1993). Nonetheless, *O. vitiosa* has become well established in Florida (Center et al., 2000). Several factors may contribute to its success, among them is their integumental terpenoid defense.

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ANTIMICROBIAL ACTIVITY OF EXOCRINE GLANDULAR SECRETION OF *Chrysomela* LARVAE

JÜRGEN GROSS,* LARS PODSIADLOWSKI, and MONIKA HILKER

Institut für Biologie
Freie Universität Berlin
Grunewaldstr. 34, D-12165 Berlin, Germany

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Abstract—The exocrine glandular secretions of leaf beetle larvae of the taxon *Chrysomela* are well-known defensive devices used against some generalist predators. Salicylaldehyde is the major repellent component of secretions emitted by larvae of *Chrysomela vigintipunctata* and *C. lapponica*, which feed on salicin-rich Salicaceae. In this study, we examined whether salicylaldehyde is also active against the entomopathogenic fungus *Metarhizium anisopliae*. The germination and growth of this fungus was strongly inhibited when salicylaldehyde was applied directly onto the blastospores. The salicylaldehyde concentration of the larval secretions of the tested willow feeding *Chrysomela* larvae was much higher than the one necessary to display this antifungal activity. Additionally, salicylaldehyde was shown to reduce germination and growth of *M. anisopliae* via the gas phase over a distance of 45 mm. Further studies on the antimicrobial activity of the salicylaldehyde-containing secretions of *Chrysomela* larvae revealed that they act nonspecifically against prokaryotic (*Escherichia coli*) and eukaryotic cells (*Saccharomyces cerevisiae* and *Trichoplusia ni*). All antimicrobial and cytotoxic effects detected proved to be due to salicylaldehyde. The larval secretions of the birch-feeding allospecies of *C. lapponica*, that do not contain salicylaldehyde, but mainly carboxylic acids and their esters, showed no detectable effects on bacteria or fungi and no cytotoxic effects against insect cells. The results are discussed with respect to their ecological relevance.

Key Words—*Chrysomela vigintipunctata*, *Chrysomela lapponica*, larval secretion, antimicrobial activity, cytotoxicity, entomopathogens, *Metarhizium anisopliae*, salicylaldehyde, carboxylic acids, carboxylic esters.

*To whom correspondence should be addressed. E-mail: jugross@zedat.fu-berlin.de

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INTRODUCTION

Larvae of the leaf beetle taxon *Chrysomela* discharge a defensive secretion from nine pairs of dorsal glands when disturbed. *C. vigintipunctata* and *C. lapponica* larvae feeding upon willows (Salicaceae) use phenolic glycosides (e.g., salicin) from their host plants as precursors to produce salicylaldehyde, the main component of their secretion (Wallace and Blum, 1969; Pasteels et al., 1988; Gross and Hilker, 1995). This secretion is highly effective for defense against some generalist predators (Pasteels et al., 1986; Palokangas et al., 1992; Rank et al., 1996). While mammals feeding upon salicin-rich willows and poplars metabolize salicylalcohol, the aglycone of salicin, to salicylic acid, (Nahrstedt, 1990), *Chrysomela* larvae oxidize salicylalcohol to salicylaldehyde (Pasteels et al., 1983). Salicylic acid is known as an antimicrobial agent (Falbe and Regitz, 1991), but no detailed knowledge is available about the antimicrobial activity of the respective aldehyde.

In *C. lapponica*, both an allospecies feeding upon Betulaceae and one feeding upon Salicaceae are known (Gross, 1997). Larvae of birch- and alder- (Betulaceae) feeding *Chrysomela* species produce isobutyric and 2-methylbutyric acids and esters of these in their glands. Like the secretions containing salicylaldehyde, this secretion acts as repellent against generalist predators (Blum et al., 1972; Hilker and Schulz, 1994).

In this study, we examined whether the larval secretions of *C. vigintipunctata* and *C. lapponica* with salicylaldehyde as major component show activity against fungal disease in addition to their repellent character against generalist predators. Furthermore, the larval secretion of the *C. lapponica* allospecies feeding upon Betulaceae was tested for its effects against fungal pathogens. The entomopathogenic fungus *Metarhizium anisopliae*, that we used in our assays, is well known as one of the most frequently occurring fungal pathogens of Chrysomelidae (Humber, 1996). Larvae of *C. vigintipunctata* and *C. lapponica* not only release their secretions when attacked by predators, but also emit secretions when disturbed by conspecific larvae or when host plant leaves are fluttered by the wind. Thus, they almost live in a cloud of volatiles produced by their own secretions. The volatiles of the secretion might prevent fungal spores from germinating on, and penetrating into, the larval cuticle. Therefore, we tested salicylaldehyde as the major component of the exocrine secretion of willow feeding larvae for its antifungal activity via the gas phase.

In order to evaluate the range of activity of the larval secretions towards microorganisms, we examined not only the activity of the secretion towards a fungal entomopathogen, but also the effects of the secretions towards other microorganisms, such as yeast and bacteria. Furthermore, we studied whether the secretions exhibit cytotoxic effects against insect cells.

METHODS AND MATERIALS

Leaf Beetles. Larval secretions containing salicylaldehyde as the main component were obtained from willow-feeding third-instar larvae of *Chrysomela vigintipunctata* and *C. lapponica*. Larvae of *C. vigintipunctata* were collected from *Salix fragilis* in the area of Marianske Lazne, Czech Republic. *C. lapponica* larvae were collected from *S. borealis* in Finnish Lapland near the Kevo Subarctic Research Institute. Larval secretion containing no salicylaldehyde, but carboxylic acids and their esters were obtained from birch-feeding second- and third-instar larvae of *C. lapponica*. They were collected in the Czech Republic from *Betula pubescens*. Larvae were reared in the laboratory in small plastic boxes ($15 \times 15 \times 6$ cm) at 15°C and a 16L:8D photoperiod (Czech specimen) or constant light (Finnish specimen). They were fed with leaves of their natural host plants.

Samples. For collecting exocrine secretions from larvae for chemical analyses and antimicrobial assays, larvae were disturbed with forceps. The secretory fluid emerging from the glandular openings was collected using sterile glass capillaries (samples for GC-MS analyses and microtiter plate experiments) or small disks of sterile filter paper (5 mm diam., samples for assays on agar plates). For some experiments, synthetic salicylaldehyde (Roth) was used instead of the larval secretion.

GC-MS Analysis. The chemical composition of larval secretions from *C. vigintipunctata* and *C. lapponica* was analyzed in earlier studies (see Introduction for references). Nevertheless, we analyzed the chemical composition of larval secretions from those populations that were examined for their antimicrobial activity, since the chemical composition of larval secretions may vary between populations of a species and between the host plants the larvae fed upon (e.g., Rowell-Rahier and Pasteels, 1986; Gross and Hilker, 1995; Soetens et al., 1998). Coupled gas chromatography–mass spectrometry was used for analyses. Secretions that were stored in calibrated glass capillaries were dissolved in specific volumes of dichloromethane. Dodecane (0.1 μg/μl; Aldrich) was added as an internal standard. One microliter of each solution was injected splitless into a gas chromatograph (Fisons GC 8060, Thermoquest, injector temperature 240°C), equipped with a 30 m $\times$ 0.32 mm $\times$ 0.25 μm DB-5 column (Fisons). The temperature program started at 40°C, was held for 4 min, and raised to 280°C at 10°C/min. Helium (Air Liquide) was used as the carrier gas (inlet pressure 10 kPa). The gas chromatograph was coupled to a quadrupole mass spectrometer (Fisons MD 800; Thermoquest). EI mass spectra were recorded at 70 eV. Structures of the identified components were confirmed by coinjection of synthetic samples. The following secretion components were quantified: benzaldehyde (Fluka); salicylaldehyde (Roth); 2-phenylethanol (Sigma); hexyl isobutyrate, (*Z*)-3-hexenyl isobutyrate, and hexylbutyrate (Aldrich); and 2-phenylethyl isobutyrate and 2-phenylethyl

2-methylbutyrate (synthesized as described by Hilker and Schulz, 1994; purity >98%). Quantitative data for each component were obtained by analyses of the secretions from five larvae of each secretion type considered in this study (*C. lapponica* on birch, *C. lapponica* on willow, *C. vigintipunctata* on willow).

Assay of Cytotoxicity Towards Insect Cells: MTT Assay. The cytotoxicity of larval secretions and pure salicylaldehyde towards insect cells was determined using the MTT assay as designed by Mosmann (1983). We used cells of *Trichoplusia ni* (Lepidoptera: Noctuidae). In MTT assays, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma) is used as coloring agent for detection of live fungal cells, as described below. To conduct this assay, cells were grown in Grace's insect medium (GIM, Life Technologies) supplemented with 10% fetal bovine serum. A volume of 5 ml of a culture with an initial cell number of 2×10^5 cells/ml was filled into a culture-flask (25 cm^2 cultivation area), and subcultured every two to three days. To determine cytotoxic effects of the samples, each 200 μl of a cell suspension was transferred into wells of one row of 96-well microtiter plates. The cell number was adjusted to 5×10^4 cells/well. After one day, the cells were attached at the bottom of the plate and medium was replaced by 200 μl test solution (= GIM containing 0.05 μl larval secretion or different concentrations of salicylaldehyde: 11.7, 1.17, 0.12, and 0.01 μg/μl; these concentrations are equivalent to 10, 1.0, 0.1, and 0.01 μl salicylaldehyde/ml medium). The microtiter plates were covered by a lid that left about 0.75 mm between samples and lid. To quantify the distance over which volatiles of larval secretions or salicylaldehyde show cytotoxic effects, one row at 9 mm distance, and a second row at 90 mm distance from the wells containing test solutions were filled with cells and medium. Each test solution was assayed on a separate plate, as the volatile samples also affect neighboring wells. For control, 200 μl GIM was applied as above. After 24 hr of incubation at room temperature, 20 μl MTT solution (5 mg/ml medium) was added and incubation was continued for 4 hr. The yellow MTT invades into the cells. The tetrazolium ring of MTT is cracked by mitochondrial dehydrogenases that are active only in living cells. Blue crystals of formazan are formed. The medium was then replaced by 100 μl isopropanol (Merck) containing 0.1% HCl (Merck) and incubated for 10 min at room temperature. Isopropanol destroys the cells and dissolves formazan. Absorbance of the resulting blue solutions was read at 560 nm using a microtiter plate reader (Titertek).

Assaying of Antibacterial Activity. Lytic Zone Assay. Lytic activity of larval secretions and salicylaldehyde was monitored with a lytic zone assay against *Micrococcus luteus*. Agar plates were prepared according to Mohrig and Messner (1968): A volume of 5 ml of sterile *M. luteus* agar was added to Petri dishes (9 cm diam.) (1% Agar L11, Oxoid; 5 mg/ml freeze-dried *M. luteus*, Sigma; 0.1 mg/ml streptomycin sulfate, Serva; in 67 mM potassium phosphate buffer, pH 6.4). Samples were applied onto disks of sterile filter paper (5 mm diam.) that were placed on the agar surface. The tested samples per filter paper disk were (a) secretion of

one larva of *C. vigintipunctata* or *C. lapponica* of each allospecies. The secretion was directly sucked onto the filter paper when the larva released the secretion after disturbance; (b) 5 μl of salicylaldehyde dissolved in dichloromethane (0.5 μl salicylaldehyde in 4.5 μl dichloromethane), and (c) 5 μl dichloromethane for control. Due to the high volatility of the samples, each sample was placed on a different agar plate. During incubation, the turbid agar was cleared by lytic activity of the samples forming clear zones. After a 24-hr incubation at 37°C, filter paper disks were removed. It was recorded whether clear zones appeared at the places where filter paper disks had been located.

Inhibition Zone Assay. Activity of larval secretions and salicylaldehyde against *Escherichia coli* was measured by an inhibition zone assay using a lipopolysaccharide-defective, streptomycin- and ampicillin-resistant mutant of *E. coli* K12 strain D31 (Faye and Wyatt, 1980). Petri dishes (9 cm diam.) were filled with 5 ml agar suspension (2.5% nutrient broth I, Merck; 1% Agar L11, Oxoid; and 0.1 mg/ml streptomycin sulfate, Serva) containing 2×10^4 viable bacteria. Samples were applied at the same concentrations and the same conditions as described above for lytic zone assay. After incubation of the agar plates (24 hr, 37°C), filter paper disks were removed and diameters of inhibition zones were determined.

Antifungal Activity. Inhibition Zone Assay. Toxic effects of larval secretions and salicylaldehyde towards yeast were quantified by an inhibition zone assay (Vilcinskas and Matha, 1997). Yeast (*Saccharomyces cerevisiae*, strain 211) was grown in a liquid medium containing 0.2% glucose (Merck), 0.1% yeast extract (Difco Laboratories) and 0.05% peptone (Sigma) for 24 hr at 37°C. Test plates were prepared as described by Gross et al. (1998). Then 1 g agar (Agar Bacteriological No. 1, Oxoid) was dissolved in 100 ml yeast medium, 1 ml sodium phosphate buffer (0.1 M, pH 7) was added, and the medium was sterilized. Finally, 2 ml of yeast suspension was added to the medium and 5 ml was filled in each Petri dish (9 cm diam.). Samples were applied at the same concentrations and same conditions as described above for the lytic zone assay. Inhibition zones were determined after 24 hr at 37°C.

Colorimetric Fungus Assay. In order to study whether salicylaldehyde affects the germination ability of blastospores and the growth and survival of hyphae of *M anisopliae*, we modified the MTT assay (see above). Conidia from stationary cultures of *Metarhizium anisopliae* (strain 8C-D2202) were used to inoculate nutrient broth (25 g Standard I, Merck, and 20 g glucose, Merck, dissolved in 1 liter distilled water and sterilized). Blastospores were harvested after four to six days of incubation, using a miracloth filter (Calbiochem) to remove mycelia. Blastospores were washed by centrifugation and resuspended in fresh medium. The density was determined (Bürker counting chamber) and adjusted to 2×10^6 spores/ml. The wells of a 96-well microtiter plate were filled with 100 μl (2×10^5 spores) of this suspension. After 30 min the blastospores had sedimentated to the bottom and medium was exchanged with 200 μl fresh medium containing

0.5 mg/ml MTT and different concentrations of salicylaldehyde (11.7 μg/μl, 1.17 μg/μl, 0.12 μg/μl, and 0.01 μg/μl; these concentrations are equivalent to 10 μl, 1.0 μl, 0.1 μl, and 0.01 μl salicylaldehyde/ml medium). For control, only 200 μl of medium containing 0.5 mg/ml MTT was applied under the same conditions as described above. With respect to the volatility, samples were always tested on separate plates and all plates were covered with lids (distance between lid and plate: 0.75 mm). The plates were incubated for 48 hr at room temperature. During this time blastospores could germinate and grow hyphae. After incubation, the medium was replaced with 200 μl isopropanol (Merck) containing 0.1% HCl (Merck). The hyphae in each well were mechanically disrupted by mixing the fluid with a 200-μl pipet. After a 10-min incubation at room temperature, 100 μl from each well was transferred to empty wells of a fresh microtiter plate. As described above, the staining agent MTT colors only living cells. Absorbance was read at 560 nm using a microtiter plate reader (Titertek).

Quantification of Distance Effects: Colorimetric Fungus Assay. To quantify the antifungal effect of volatile salicylaldehyde over distance, we modified the colorimetric fungus assay with *M. anisopliae*. In one row of a 96-well microtiter plate; each of six wells was filled with 0.5 μl salicylaldehyde, all others were filled with 200 μl blastospore suspension (2×10^5 spores/well) in fresh medium (see Colorimetric Fungus Assay) with MTT (0.5 mg/ml). The plate was covered with a lid, incubated for 48 hr at room temperature and viability was determined as described above. To prevent errors due to edge effects, the viability of the blastospores in edge wells was not recorded.

Statistical Analysis. Differences between the concentrations of the quantified compounds of larval secretions, between the diameters of lysis and inhibition zones, and between absorbance measured in fungal colorimetric assays were evaluated by the Mann-Whitney U test (Sachs, 1992). Multiple comparisons of data were confined to Bonferroni correction. Differences between absorbance of colored suspensions of the cells in the MTT test for cytotoxicity were evaluated by one-way ANOVA and post-hoc Scheffé tests.

RESULTS

Composition of Larval Secretions and Quantification of Constituents. The composition of larval secretions of *C. vigintipunctata* feeding upon *S. fragilis* did not differ from the literature (Pasteels et al., 1982; Soetens et al., 1998). The secretion is composed mainly of salicylaldehyde and shows traces of benzaldehyde (Table 1). GC-MS analyses of the larval secretion of *C. lapponica* feeding either upon *B. pubescens* or *S. borealis* also revealed the presence of known components for these species. Secretions of *C. lapponica* larvae feeding upon birch contained neither benzaldehyde nor salicylaldehyde, but did contain isobutyric and

TABLE 1. CONCENTRATIONS OF COMPOUNDS IN THREE DIFFERENT SECRETION TYPES OF *Chrysomela* Larvae ($N = 5$) DETECTED AND QUANTIFIED BY GC-MS ANALYSES[a]

	Concentration (μg/μl, mean $\pm$ SD)		
	C. lapponica		*C. vigintipunctata*
Compound	*Betula*	*Salix*	*Salix*
Benzaldehyde	0	0.20 ± 0.21^a	0.07 ± 0.02^a
Salicylaldehyde	0	58.36 ± 31.08^a	81.50 ± 9.68^a
2-Phenylethanol	0	0.11 ± 0.11	0
Hexyl isobutyrate	0.76 ± 0.12^a	0.01 ± 0.01^b	0
(*Z*)-3-Hexenyl isobutyrate	0.88 ± 0.03	0	0
2-Phenylethyl isobutyrate	0.84 ± 0.11^a	0.05 ± 0.05^b	0
2-Phenylethyl 2-methylbutyrate	1.01 ± 0.14^a	0.02 ± 0.02^b	0

[a] Different letters indicate significant ($P < 0.05$) differences between columns. Mann-Whitney U test.

2-methyl butyric acid and several esters. Secretions of *C. lapponica* larvae feeding on *S. borealis* contained salicylaldehyde as the major component and small amounts of benzaldehyde and carboxylic acid esters (Table 1) (Hilker and Schulz, 1994; Gross, 1997).

Direct and Distance Effects of Cytotoxicity. The results of the cytotoxicity assays with the larval secretions and salicylaldehyde are presented in Table 2. When applied directly onto the cell cultures of *T. ni*, concentrations of synthetic salicylaldehyde of 0.12 μg/μl and higher exhibited significant cytotoxicity. Direct application of 0.05 μl of the *C. vigintipunctata* larval secretion also resulted in cytotoxic activity. However, cytotoxic effects were not caused by application of the secretion of birch-feeding *C. lapponica* larvae. High concentrations (11.7 μg/μl) of synthetic salicylaldehyde showed a cytotoxic effect via the gas phase over a distance of 90 mm; concentrations of 1.17 and 11.7 μg/μl also caused a cytotoxic effect over a distance of 9 mm compared to the respective control. The *C. vigintipunctata* larval secretion inhibited the growth of insect cells over a distance of only 9 mm.

Antibacterial Activity. Neither salicylaldehyde nor the larval secretions showed activity in the lytic zone assay with the gram-positive bacterium *Micrococcus luteus* (Table 3). In contrast, secretions of the willow-feeding leaf beetle larvae showed a significant growth inhibition of the gram-negative bacterium *Escherichia coli*. The tested synthetic sample of salicylaldehyde, which is the main component of the secretions of both willow feeding leaf beetle species, had also, a strong inhibitory effect. The larval secretion of the birch-feeding allospecies of *C. lapponica*, which does not contain any salicylaldehyde, caused no growth inhibition of *E. coli* (Table 3).

Antifungal Activity. The antifungal activity of larval secretions and salicylaldehyde as measured by the inhibition zone assay with the yeast *Saccharomyces*

TABLE 2. RESULTS OF MTT ASSAY FOR QUANTIFICATION OF CYTOTOXICITY OF EXOCRINE SECRETION OF *Chrysomela* LARVAE TOWARDS CELLS OF *Trichoplusia ni*[a]

Distance to samples (mm)		Absorbance (mAbs)						
							Larval secretion [0.05 μl]	
			Salicylaldehyde [μg/μl]				*C. vigintipunctata*	*C. lapponica*
		Control	0.01	0.12	1.17	11.7	*Salix*	*Betula*
Direct	$\bar{x}$	0.40	0.47	0.24	0.15	0.17	0.13	0.32
	SD	0.12	0.09	0.06	0.02	0.05	0.02	0.07
	p	a	a	bc	bc	bc	c	ab
9	$\bar{x}$	0.40	0.45	0.42	0.21	0.20	0.24	0.31
	SD	0.12	0.09	0.07	0.03	0.05	0.03	0.07
	p	a	a	a	b	b	b	ab
90	$\bar{x}$	0.40	0.48	0.39	0.41	0.21	0.45	0.54
	SD	0.12	0.05	0.07	0.11	0.02	0.07	0.13
	p	a	a	ab	ab	b	a	a

[a]Mean values and standard deviations ($N = 6$) of photometrically measured absorbance (mAbs) of coloring produced by vital cells of *T. ni* at 0 mm (direct application), 9 mm, and 90 mm distance to wells with different concentrations of salicylaldehyde, or 0.05 μl larval secretion from *Chrysomela vigintipunctata* feeding on *Salix fragilis*, or 0.05 μl larval secretion of *C. lapponica* feeding on *Betula pubescens*. One-way ANOVA followed by Scheffé tests. Different letters indicate significant ($P < 0.05$) differences between treatments at a given distance to control. Lower absorbance values of test samples in comparison to the control mean significant reduction of living cells in test samples due to cytotoxic effects.

TABLE 3. RESULTS OF LYTIC/INHIBITION ZONE ASSAYS WITH GRAM-POSITIVE BACTERIUM *Micrococcus luteus*, GRAM-NEGATIVE BACTERIUM *Escherichia coli*, AND FUNGUS *Saccharomyces cerevisiae*[a]

Treatment	*M. luteus*	*E. coli*	*S. cerevisiae*
Control	0	0	0
Larval secretion of *C. lapponica* (*Betula*)	0	0	0
Larval secretion of *C. lapponica* (*Salix*)	0	2.3 ± 0.92^b	1.4 ± 1.00^b
Larval secretion of *C. vigintipunctata* (*Salix*)	0	3.7 ± 0.46^b	3.9 ± 0.97^b
Salicylaldehyde	0	7.4 ± 1.10^b	5.2 ± 0.81^b

[a]Mean values and standard deviations ($N = 6$) of the lytic/inhibition zone diameters caused by synthetic salicylaldehyde and by exocrine secretions of larvae of *Chrysomela lapponica* feeding upon birch (*Betula pubescens*), and of *C. lapponica* and *C. vigintipunctata* feeding upon willow (*Salix* ssp.). Glandular secretion of one larva was collected with filter paper disks and placed onto agar plates. Volumes of 5 μl salicylaldehyde dissolved in dichloromethane (0.5 μl salicylaldehyde in 4.5 μl dichloromethane) or 5 μl dichloromethane (control) were applied to filter paper disks and placed onto agar plates.

[b]Significant ($P < 0.05$) difference from control within a treatment.

cerevisiae are shown in Table 3. Germination and growth of the fungus were inhibited by both the exocrine secretions of the willow-feeding larvae and synthetic salicylaldehyde, but not by the secretion of birch-feeding larvae of *C. lapponica*.

The antifungal activity of salicylaldehyde as measured by the colorimetric fungus assay with the entomopathogenic fungus *Metarhizium anisopliae* is presented in Figure 1A. Since mitochondrial enzymes of living hyphae produce the blue color formazan from MTT, high absorbance values mean high germination and growth rates of *M. anisopliae*. A concentration of 0.12 μg/μl salicylaldehyde reduced the germination and growth of the fungus by more than 50% when compared to the control (Figure 1A). The level of salicylaldehyde in the larval secretion of willow feeding *Chrysomela* larvae is much higher (see Table 1). In order to reach such a 50% reduction of germination and growth, a much higher concentration of the common fungicide nystatin (1.0 μg/μl) was necessary (Figure 1B).

Distance Effect of Antifungal Activity. Results of the colorimetric fungus assay for quantification of a distance effect of antifungal activity of salicylaldehyde via the gas phase are given in Figure 2. At a distance of 9–36 mm from wells containing 0.5 μl salicylaldehyde, germination of blastospores of *M. anisopliae* and growth of hyphae were strongly inhibited. At a distance of 45 mm from wells containing salicylaldehyde, a weaker, but still significant reduction of fungal activity was recorded (Mann-Whitney U test, Bonferroni corrected, $P < 0.0004$). At greater distances, inhibition of fungal blastospore germination and growth could no longer be detected.

DISCUSSION

The present study shows that larval exocrine secretions from willow-feeding leaf beetles exhibited antibacterial, antifungal and cytotoxic activities. Salicylaldehyde, the major component of the secretion of willow-feeding larvae, was shown to display these activities (Figures 1 and 2; Tables 2 and 3). Larvae are protected from the cytotoxic activity of their own secretion by a cuticular lining of the glandular reservoirs that store the secretions (Garb, 1915; Hinton, 1951; Oldham et al., 1996; Gross et al., 1998).

The results of our study show that secretions of willow feeding larvae act nonspecifically against a wide range of microorganisms. We consider the antifungal activity of the exocrine larval secretions to be of particular ecological significance. Fungal attack occurs when fungal hyphae penetrate the cuticle at the larval body surface, which is where the exocrine secretion may display its antifungal activity. Glands releasing the antifungal secretion are distributed segmentally on the larval body from the mesothorax to the seventh abdominal segment and thus, may, protect

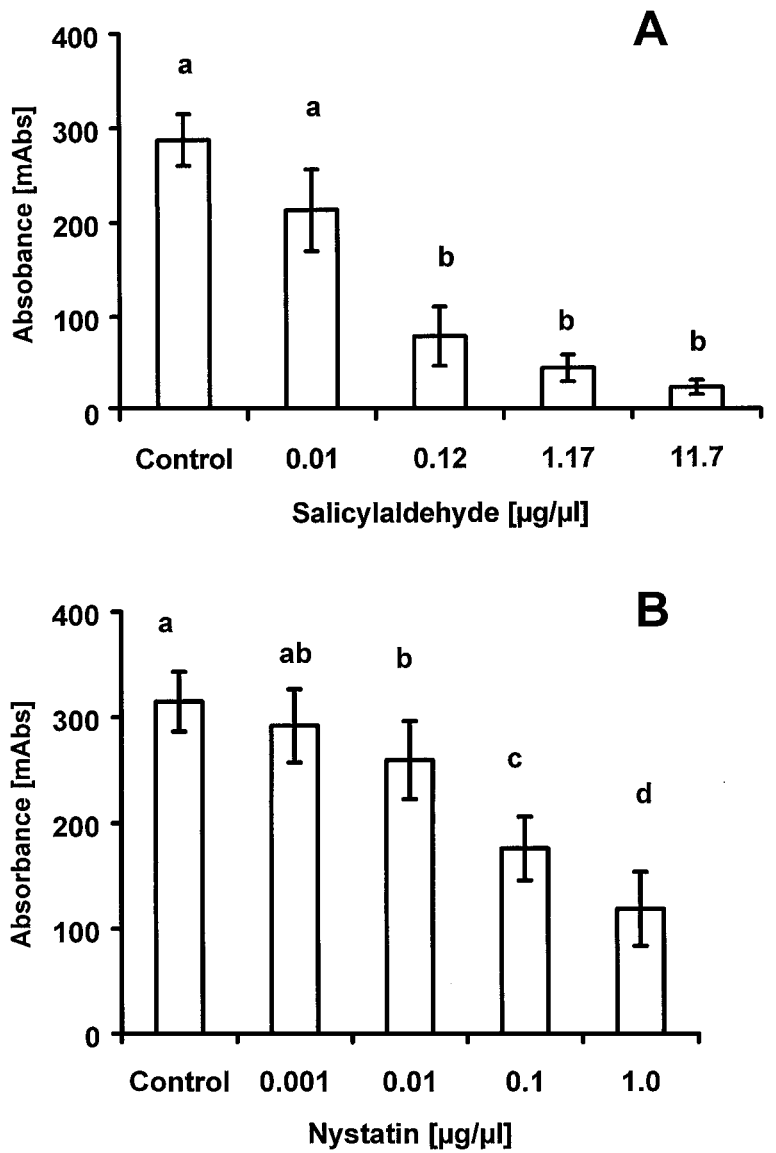


FIG. 1. Results of the colorimetric fungus assay. Mean values and standard deviations ($N = 6$) of photometrically measured absorbance (mAbs) of coloring produced by *Metarhizium anisopliae* after treatment with different concentrations ($\mu g/\mu l$) of (A) salicylaldehyde and (B) nystatin. Less coloring means reduced activity of mitochondrial enzymes and, thus, reduced blastospore germination and hyphal growth. Mann-Whitney U test (Bonferroni corrected); different letters indicate significant ($P < 0.005$) differences.

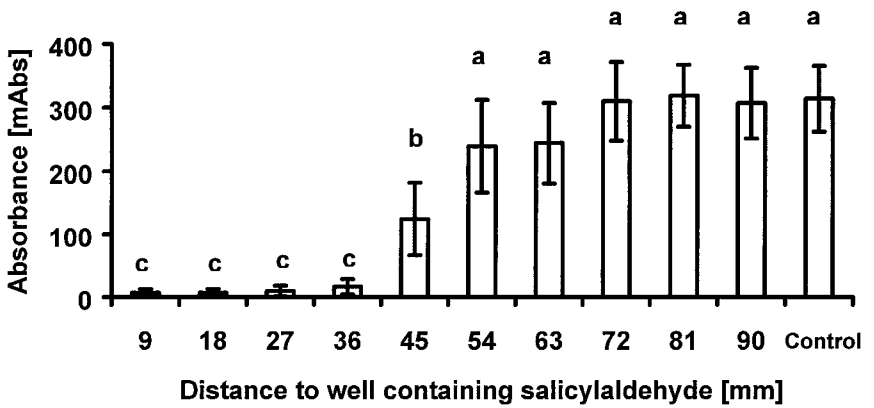


FIG. 2. Quantification of antifungal effects of salicylaldehyde via the gas phase using the modified colorimetric fungus assay with blastospores of the entomopathogenic fungus *Metarhizium anisopliae*. Mean values and standard deviations ($N = 18$) of photometrically measured absorbance (mAbs) of coloring produced by *M. anisopliae*. Distance between wells containing 0.5 μl salicylaldehyde to measured wells extend from the left to the right. Mann-Whitney U test (Bonferroni corrected); different letters indicate significant ($P < 0.0004$) differences.

a large part of the larval surface from fungal penetration. Pathogenic bacteria may only invade the larval body orally or via injuries. Larval secretions may be unable to act against bacteria at feeding sites, unless volatiles in the secretion also show activity at contaminated feeding sites.

The range of activity of the secretion via the gas phase was not tested for bacteria, but towards insect cells and the entomopathogenic fungus *M. anisopliae*. Cytotoxic activity of secretions of willow-feeding larvae was obvious when applied directly onto insect cells and was also detected over a distance of 9 mm via the gas phase (Table 2). However, salicylaldehyde exhibited antifungal activity via the gas phase over a distance of 45 mm (Figure 2). This range of activity was recorded when the microtiter plate with wells containing 0.5 μl salicylaldehyde was covered with a lid. The antifungal activity range of the secretion containing salicylaldehyde is expected to be much lower in a natural environment. Nevertheless, since paired larval glands are located on nine larval segments, even antifungal activity via the gas phase over much shorter distances might be sufficient to effectively protect these larvae from fungal attack. During larval development, *C. lapponica* or *C. vigintipunctata* larvae grow from approximately 2 mm to approximately 9 mm length.

The antimicrobial effects of the larval secretion of *C. vigintipunctata*, as measured by inhibition zone assays, were stronger than those of the secretion of

C. lapponica larvae feeding on willow (Table 3; Mann-Whitney U test: $P < 0.001$). For the inhibition zone assays, larval secretions had been collected on filter paper that was then placed onto agar plates in order to examine whether the secretion released by one larva is sufficient to show antimicrobial effects. We did not measure the volume of secretion released by each larva. The larvae of *C. vigintipunctata* from which secretion was obtained for the bioassays were larger than those of *C. lapponica* and may have released more secretion than the *C. lapponica* larvae. Even though concentrations of salicylaldehyde in larval secretions of *C. vigintipunctata* and willow feeding *C. lapponica* were not significantly different (Table 1), higher amounts of salicylaldehyde due to larger secretion volumes might explain the stronger activity of the larval secretion of *C. vigintipunctata* detected by the inhibition zone assays.

The volume of secretion and the concentration of salicylaldehyde within the secretion are known to decrease when larvae of *C. vigintipunctata* are transferred from a salicin-rich willow like *S. myrsinifolia* to *S. caprea*, a willow species with a very low concentration of phenolic glucosides (Julkunen-Tiitto, 1986; Soetens et al., 1998). Volumes (salicylaldehyde concentrations) of secretions ranged from 0.103 μl $\pm$ 0.029 (526 μg/μl $\pm$ 190) on *S. myrsinifolia* to 0.047 μl $\pm$ 0.019 (0.83 μg/μl $\pm$ 0.35) on *S. caprea* (Soetens et al., 1998). Willow beetle larvae that feed on Salicaceae with small contents of salicin suffer an increased risk of predation by some generalist predators (Matsuda and Sugawara, 1980; Pasteels et al., 1986; Palokangas et al., 1992), but they might also benefit from the low salicylaldehyde concentrations of their secretions due to a decreased rate of detection by specialist predators, that use salicylaldehyde as a kairomone for finding prey (Pasteels and Gregoire, 1984; Rank, 1994; Köpf et al., 1997; Sears et al., 2001). However, the results of the present study indicate that a reduced concentration of salicylaldehyde in the larval secretion decreases the defensive efficacy against pathogen attacks (compare Figure 1A).

In contrast to the willow-feeding allospecies of *C. lapponica*, the secretion of the birch-feeding larvae, that did not contain salicylaldehyde, was inactive against microorganisms and insect cells. These differences between secretions of birch- and willow-feeding allospecies are supported by our field observations. Fungal infections of larvae have never been observed in larvae of the willow-feeding Finnish population. In contrast, when recording larval mortality of the birch-feeding Czech population during a rainy season, 30% of the larval groups (about 30 larvae per group) were found dead with obvious fungal infection of several larvae within a group (unpublished data). During a five-year study of natural enemies of the willow-feeding allospecies of *C. lapponica* in Russia, only 2% of pupal mortality had been caused by microbial infections (Zvereva and Kozlov, 2000). Pupae may be protected against microbial infection by the salicylaldehyde-containing larval secretion that evaporates from the glandular reservoirs of the larval exuvia that remains attached to the pupa.

Only three species of the taxon *Chrysomela* feed on Betulaceae: *C. interrupta* and *C. mainensis* feed on alder (Brown, 1956), and one allospecies of *C. lapponica* feeds on birch (Letzner, 1876). The exocrine larval secretions of these *Chrysomela* species on Betulaceae are well known to repel generalist predators (Hilker and Schulz, 1994). We suppose that the larval secretions of all these *Chrysomela* species feeding on Betulaceae do not exhibit any antimicrobial effects because of their chemical similarity (Termonia and Pasteels, 1999). Further studies are needed to elucidate whether, and how, these Betulaceae feeding *Chrysomela* species balance the disadvantage of being unprotected against pathogens by other positive effects that might have favored the specialization of these beetles on birch or alder (Termonia et al., 2001).

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CHARACTERIZATION AND TOXICITY OF *Amanita cokeri* EXTRACT

DENNIS C. DREHMEL and WILLIAM SCOTT CHILTON*

Department of Botany
North Carolina State University
Raleigh, North Carolina 27695

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Abstract—The nonprotein amino acids 2-amino-3-cyclopropylbutanoic acid and 2-amino-5-chloro-4-pentenoic acid were isolated from the mushroom *Amanita cokeri*. The cyclopropyl amino acid is toxic to the fungus *Cercospora kikuchii*, the arthropod *Oncopeltus fasciatus* (milk weed bug), and the bacteria *Agrobacterium tumefaciens*, *Erwinia amylovora*, and *Xanthomonas campestris*. Toxicity to bacteria was reversible by addition of isoleucine to the medium. No toxicity was observed for 2-amino-5-chloro-4-pentenoic acid.

Key Words—Basidiomyces, *Amanita*, *Lepidella*, 2-amino-3-cyclopropylbutanoic acid, 2-amino-5-chloro-4-pentenoic acid, *Oncopeltus*, *Cercospora*, *Agrobacterium*, *Erwinia*, *Xanthomonas*.

INTRODUCTION

The mushroom genus *Amanita* Pers. per Hook. has a reputation for having a high ratio of species toxic to humans. Those of section *Phalloidae* sensu Drehmel (Drehmel et al., 1999; Moncalvo et al., 2000), containing cyclopeptide amatoxins, are the most deadly. Amanita species of section *Amanita* subsection *Amanita* sensu Drehmel are characterized by the less deadly, neurotoxic amino acids ibotenic acid and muscimol. Amanita species of section *Lepidella* (Gilb.) Corner & Bas, which lack amatoxins and neurotoxic amino acids, are characterized by the presence of unsaturated, C_5–C_7 nonprotein amino acids containing unsaturation or cyclopropyl groups and, often, chlorine (Table 1). Cyclopropyl amino acids are of interest because 2-amino-3-cyclopropylpropionic acid has been shown to be "a potent antagonist" of *Escherichia coli* (Meek and Rowe, 1955) and can inhibit germination of spores from *Pyricularia oryzae* Cav. (Ohta et al., 1986).

* To whom correspondence should be addressed. E-mail: scott_chilton@ncsu.edu

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TABLE 1. UNSATURATED AND CYCLOPROPYL AMINO ACIDS OF SECTION *Lepidella*

2-Amino acid	Source species	Reference
4-pentenoic	*pseudoporphyria*	Hatanaka et al. (1985)
4-pentynoic	*pseudoporphyria*	Hatanaka et al. (1985)
	abrupta	Yamaura et al. (1986)
5-hexenoic	*gymnopus*	Hatanaka (1992)
4-hexynoic	*pseudoporphyria*	Hatanaka (1992)
	miculifera	Hatanaka et al. (1998)
6-hydroxy-4-hexynoic	*miculifera*	Hatanaka et al. (1998)
5-hexynoic	*gymnopus*	Hatanaka (1992)
4,5-hexadienoic	*solitaria*(=*smithiana*)	Chilton et al. (1968)
	pseudoporphyria	Hatanaka (1975)
	neoovoidea	Hatanaka and Kawakami (1980)
	abrupta	Yamaura et al. (1986)
		Ohta et al. (1987)
	gymnopus	Hatanaka et al. (1992)
	miculifera	Hatanaka et al. (1998)
hept-4-en-6-ynoic	*pseudoporphyria*	Hatanaka et al. (1985)
4-chloro-4-pentenoic	*pseudoporphyria*	Hatanaka et al. (1974)
5-chloro-4-pentenoic	*virgineoides*	Ohta et al. (1995)
	castanopsidis	Yoshimura et al. (1999)
	cokeri	This study
5-chloro-4-hexenoic	*solitaria* (=*smithiana*)	Chilton and Tsou (1972)
5-chloro-6-hydroxy-4-hexenoic	*abrupta*	Ohta et al. (1987)
5-chloro-5-hexenoic	*miculifera*	Hatanaka et al. (1998)
5-chloro-4-hydroxy-5-hexenoic	*gymnopus*	Hatanaka et al. (1994)
	sp./section *Lepidella*	Hatanaka et al. (1995)
3-cyclopropylpropionic	*virgineoides*	Ohta et al. (1986)
	polypyramis	Chilton and Drehmel (2001)
3-cyclopropylbutanoic	*castanopsidis*	Yoshimura et al. (1999)
	cokeri	Chilton and Drehmel (2001)

Very little is known about the edibility or human toxicity of species in section *Lepidella* or whether nonprotein amino acids of this section play a role in defense of the carpophore. Earlier studies have shown toxicity of 2-amino-4,5-hexadienoic acid from *A. smithiana* to guinea pigs (Chilton and Tson, 1973). The amino acid fraction of *A. smithiana* also damages renal epithelial cells (Pelizzari et al., 1994). The amino acid fraction of *A. abrupta*, containing 2-amino-4,5-hexadienoic acid and 2-amino-4-pentynoic acid, also causes liver damage in mice (Yamaura et al., 1986). The 2-amino-4-chloro-4-pentenoic acid isolated from *A. pseudoporphyria* had antibacterial activity against a number of human pathogens (Moriguchi et al., 1987).

A common mushroom of section *Lepidella* in the southeastern United States is *Amanita cokeri* (Gilb. & Kuhner) Gilb. In view of the previously reported

biological activity of nonprotein amino acids of section *Lepidella*, we have tested potential antimicrobial and antiinsect defensive effects of the two major nonprotein amino acids of this mushroom, 2-amino-3-cyclopropylbutanoic acid (Chilton and Drehmel, 2001) and 2-amino-5-chloro-4*E*-pentenoic acid, chemically characterized in the present study.

METHODS AND MATERIALS

General Characterization. Fresh fruiting bodies of *A. cokeri* were collected in August 1998 in Wake County, North Carolina (Duke University herbarium number HN5914). They were processed by brushing away dirt, weighed, and extracted in a blender with 70% ethanol in water. A 1-ml sample of the extract was evaporated to dryness in a Speed Vac to determine the solids concentration. The solids were redissolved in distilled water to achieve a standard of 0.1 mg/μl. Amino acids of these samples were analyzed by cellulose thin-layer chromatography (TLC), paper chromatography (PC), and high-voltage paper electrophoresis (HVPE). TLC and PC were developed with 1-butanol–water–acetic acid (BAW, 12:3:5). Amino acids were detected by ninhydrin reagent.

Isolation of Amino Acids. Total amino acids in mushroom extracts were absorbed on a Dowex $50H^+$ column (1×10 cm) and eluted with dilute ammonia. The total amino acid fraction was further fractioned by descending preparative PC (BAW 12:3:5), allowing the solvent front to drip off the end of the paper for better resolution. Individual amino acids of interest were redissolved off of the PC. The purity of the amino acids isolated was verified by TLC or PC and by HVPE using ninhydrin detection. The structure of the nonprotein amino acids isolated was verified by NMR and mass spectroscopy.

Toxicity Tests. Standard test solutions of both total and individual amino acids were prepared to a concentration of 0.1 mg/μl. Standard solutions were also prepared of two well-studied toxins from plants: DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), from corn seedlings, and tenulin, a sesquiterpene lactone from bitter sneeze weed (*Helenium amarum* [Raf.] H. Rock). All of these standard solutions, including the standard solution of the unrefined extract, were used in three types of toxicity tests that were targeted toward bacteria, fungi, and arthropods.

Bacteria. The bacterial organisms used were *Agrobacterium tumefaciens* (Smith & Townsend) Conn, *Erwinia amylovora* (Burrill) Winslow, Broadhurst, Buchanan, Krumwiede, Rogers & Smith, and *Xanthomonas campestris* pv. *zinniae* (Pammel) Dawson. A liquid culture of the bacteria and soft agar was applied to a culture plate of hard agar incorporating nutrient broth. Treated 3MM paper disks were applied, and the zone of inhibition was observed after 48 hr. The paper disks (7 mm diam.) were treated in a two-step process. First, to each disk was applied

10 μl of 95% ethanol, which was allowed to dry in the sterile laminar flow hood. Second, to each disk was applied 1 μl (100 μg of test substance), 3 μl (300 μg) or 10 μl (1 mg) of a test solution, which was also allowed to dry. Each culture plate had a negative control disk that had been treated with ethanol only. Many culture plates had one or two positive control disks treated with DIMBOA or tenulin.

Fungi. Toxicity to the fungal plant pathogen *Cercospora kikuchii* (Matsumoto & Tomoyasu) M. W. Gardner was studied in two ways. In one method, the same general approach as with the bacteria was used. Instead of a liquid culture of bacteria, plugs of *Cercospora* were dispersed into 1 ml of sterile distilled water using glass beads in a glass tube vibrated by a Vortex machine. The resultant hyphal fragment inoculum was pipetted onto a potato dextrose agar (PDA) culture plate and allowed to dry in a sterile laminar flow hood. Treated disks were applied as above with the bacterial testing. In a second method, plugs were removed from a PDA plate and replaced with plugs of *Cercospora*. Again, test disks were treated and placed on the PDA plate, but in this case they were applied exactly on top of the *Cercospora* plugs.

Insects. Milkweed bugs (*Oncopeltis fasciatus* Dallas) in the third or fourth instar, or newly hatched, were housed in a 1-liter flask covered with a paper towel and supplied with raw, shelled suflower seeds and water in a 1.5-ml microcentrifuge tube fitted with a paper wick. Tested substances were dissolved in the water supply. A negative control group with no test substance was used in each experiment. In tests with third or fourth instar insects, a positive control was used with DIMBOA added to the water. Dosing schedules are noted below. With medium sized insects, the effects of both short- and long-term water deprivation also were studied. In one test with insects of various ages, the insects were given a choice between treated and untreated water to gauge the relative appeal of each.

RESULTS

Both TLC and PC of the total amino acid fraction of *Amanita cokeri* showed the presence of two prominent high R_f, low-polarity amino acids in the valine–leucine region of the chromatogram. We have previously shown (Chilton and Drehmel, 2001) that the higher R_f amino acid giving a normal ninhydrin reaction is 2-amino-3-cyclopropylbutanoic acid. Treatment of the lower R_f amino acid with ninhydrin gave an initial yellow-brown color, shifting gradually to purple, characteristic of β,γ- and γ,δ-unsaturated amino acids. The lower R_f amino acid was purified by ion exchange chromatography and preparative PC. Positive FAB MS gave a pseudo molecular ion doublet at $M + H = 150$, 152 in the 3:1 ratio of a mono-chloro compound. Similarly, negative ion FAB MS gave $M - H = 148$, 150, indicating that the elemental composition must be $C_5H_8ClNO_2$. The NMR

TABLE 2. ZONE OF INHIBITION RESULTS FOR BACTERIA (DIAMETER OF ZONE IN MILLIMETERS)

Bacteria tested	Unfractionated extract (1 mg)	Cyclopropy/AA (0.3 mg)	DIMBOA (1 mg)	Tenulin (1 mg)
Agrobacterium tumefaciens	25	30	17	21
Erwinia amylovora	26	28	nt	20
Xanthomonas campestris	14	16	nt	nt

spectrum in D_2O (α-CH, 3.64 ppm, t; β-CH_2, 2.64 ppm, q; γ-CH, 5.63 ppm, q; δ-CH 6.15 ppm, d, $J = 10$ Hz) indicates that this compound is 2-amino-5-chloro-4*E*-pentenoic acid, previously isolated by Yoshimura et al. (1999) from *A. castanopsidis*.

Results of the zone of inhibition (ZOI) tests using three different bacteria, *A. tumefaciens*, *E. amylovora*, and *X. campestris* pv. *zinniae*, were positive for the unfractionated standard extract, the total amino acid fraction, and purified 2-amino-3-cyclopropylbutanoic acid (Table 2). However, purified 2-amino-5-chloro-4-pentenoic acid did not show any zone of inhibition. For all three bacteria tested, it was possible to reverse the toxicity of 2-amino-3-cyclopropylbutanoic acid by adding isoleucine to a disk in close proximity to the disk with 2-amino-3-cyclopropylbutanoic acid. The toxicity reversal was evident in the space around the isoleucine and between the two disks but did not extend beyond this space. 2-Amino-3-cyclopropylbutanoic acid remained toxic in the space on either side of its disk and in the space away from the disk with isoleucine. This demonstrates that 2-amino-3-cyclopropylbutanoic acid acts as an antimetabolite with respect to isoleucine (Scannell and Pruess, 1975). Neither leucine nor valine demonstrated any zone of reversal in the double disk test.

Results for the test on the PDA plate with the dispersed fungus *Cercospora kikuchii* showed that there was no zone of inhibition using either unfractionated amino acids or 2-amino-3-cyclopropylbutanoic acid from *A. cokeri*. However, in the test where the treated disk was placed on top of the fungal plug, the fungal growth area at the 2-amino-3-cyclopropylbutanoic acid-treated disk was less than half (46%) of the fungal growth area at the untreated disk after a five-day period.

The unfractionated extract of *A. cokeri* was lethal to both milkweed bug neonates and later instars. In the case of the former, four populations of approximately 25 eggs each were split into two groups where one received untreated water and the other received water with 19 mg of unfractionated dried extract dissolved in 1.5 ml water in a microcentrifuge tube. Water was replenished as needed, usually every day or two to make up for consumption and evaporation. On day 5 and day

10, the resupply contained additional extract for the test group in the same dose as the initial dose. Thus, the total possible dose to the end of the early development test group was 57 mg. However, as noted below, many did not survive long enough to be exposed to this amount. At one week, there were differences between the two groups. The control group was bigger and more active, and more of the insects had progressed to later instars. Many in the test group were motionless. On day 10 before the third treatment with the extract, at least half of the test group was dead. At the two week mark, most of the test group were dead. The untreated group was mostly in the third instar and very large compared to the remaining couple of insects in the test group. All treated milkweed bugs (population = 49) were dead by day 18. Meanwhile, the untreated group was entirely healthy and proceeding to the fourth instar. To check whether death was due to dehydration, because the test insects found the treated water undesirable, approximately 50 insects were given the choice between treated and untreated water after being without water for two days. The numbers of insects at each water supply were counted every 15 min. The total number of visits to the treated water was slightly higher than that for untreated water.

As another check on possible antifeedant properties of *A. cokeri* amino acids, a total of 34 milkweed bugs of third and fourth instar were tested in five groups. There were six in a negative control group (no treatment), seven in a positive control group (treatment with DIMBOA), seven in the test group given *A. cokeri*-treated water, seven in a group given no water at all, and 7 in a group given no water for six days and untreated water after that. The DIMBOA group received one treatment of 9 mg in the water, and the test group received two treatments (day 0 and day 5) of 33 mg each in the water for a total dose of 66 mg. In all cases other than day 5 of the test group, the resupply water given every day or two was only water. After one week, six of seven of the DIMBOA group were dead, and all in the other groups were alive, although the test group with *A. cokeri*-treated water appeared lethargic. After a week and a half, all of the group denied water were dead, although prior to death many of the insects remained active and were climbing the walls of the flask.

After two weeks, the group with the *A. cokeri*-treated water had difficulty walking and in some cases even had difficulty in standing. Movement was sluggish and awkward or not at all. All of the untreated group had achieved full adulthood, while only two of the test group had. All bugs in the group that had been denied water for six days only were active. After three weeks, all of the untreated group remained active adults, in contrast to the *A. cokeri*-treated group where only one survived. The bugs in the group denied water for only six days remained active and showed no signs of difficulty in movement as was seen with the *A. cokeri*-treated group. Of the lethal conditions used in testing medium-sized insects, the treatments may be compared by the time to greater than 50% death of the test population: for DIMBOA, 4 days; for no water, 9 days; for *A. cokeri* extract, 17 days.

DISCUSSION

The hallmark of the nonprotein amino acids of *Amanita* section *Lepidella* is the presence of unsaturated norvalines and norleucines. Hatanaka (1992) has suggested that all of the acyclic unsaturated and chlorinated C-5 amino acids of Table 1 may arise by desaturation of norvaline followed, in some cases, by hydrogen chloride addition and hydroxylation. The C-6 series may be related to the C-5 amino acids by α-homologation of the type that converts the valine precusor α-ketoisovaleric acid into the leucine precursor α-ketoisocaproic acid in primary metabolism (Lehninger, 1975). The 3-cyclopropylpropionic acid could arise via one carbon addition across the double bond of 2-amino-4-pentenoic acid. However 3-cyclopropylbutanoic acid of *A. cokeri* must have a different origin.

The four microorganisms used to test toxicity of the two nonprotein amino acids of *A. cokeri* are common soil or phylloplane-inhabiting bacteria and fungi not known to be pathogenic or saprophytic on *A. cokeri*. Consequently, they were considered unlikely to have evolved resistance to the unusual amino acids of this mushroom. While no toxicity was detected for 2-amino-5-chloro-4-pentenoic acid, 2-amino-3-cyclopropylbutanoic acid inhibited growth of all four microorganisms. Toxicity of this amino acid was reversed in each case by isoleucine but not by leucine or valine. This suggests that replacement of the isoleucine β-ethyl group by a β-cyclopropyl group converts it into an isoleucine antimetabolite.

The milkweed bug, a narrow host range insect, is also unlikely to be adapted to defensive compounds of *A. cokeri*. Colonies of this insect are commonly maintained indefinitely on a more readily available diet of sunflower seeds. Addition of the 2-amino-3-cyclopropylbutanoic containing amino acid fraction of *A. cokeri* to their drinking water caused the death of all insects prior to the reproductive stage. This may be due to an antimetabolte mechanism similar to that observed with the microorganisms or by reduced water intake caused by the insects' perception of the added amino acid, or by a combination of these mechanisms. While there may be some antifeedant effects underlying the lethality of the amino acid fraction of *A. cokeri*, direct toxicity is implied by four observations. Both small and larger sized insects were observed to drink frequently and on a regular basis from treated water. When given a choice between treated and untreated water, the insects chose each in nearly equal numbers. In the tests with older insects, death by dehydration was much faster than death of insects given water containing *A. cokeri* amino acids. *A. cokeri*-treated insects demonstrated a difficulty in walking and climbing not seen in either short- or long-term dehydrated insects.

We conclude that aqueous ethanolic extracts of *A. cokeri* contain at least two nonprotein amino acids, 2-amino-3-cyclopropylbutanoic acid and 2-amino-5-chloro-4-pentenoic acid, both of which have been isolated previously form *A. castanopsidis* (Yoshimura et al., 1999). Aminocyclopropylbutanoic acid was

toxic against the three bacteria, a fungus, and an insect used in the bioassays. Toxicity toward the microorganisms is probably due to this cyclopropyl amino acid acting as an isoleucine antimetabolite. The total amino acid fraction of *A. cokeri* is lethal to milk weed bugs (*Oncopeltus fasciatus*) in both early and late stages of development. The mechanism for this is direct toxicity rather than as a drinking deterrent causing death by dehydration. Since the amino acids were not fractionated in this test, it is not known whether one of these two nonprotein amino acids is responsible, or possibly, some other, unrecognized component of the extract.

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VARIATIONS IN CAULERPENYNE CONTENTS IN *Caulerpa taxifolia* AND *Caulerpa racemosa*

OLIVIER DUMAY,[1,*] GÉRARD PERGENT,[1]
CHRISTINE PERGENT-MARTINI,[1] and PHILIPPE AMADE[2]

[1]*Équipe Écosystèmes Littoraux, Université de Corse*
FST, BP 52, 20 250 Corte, France
[2]*CNRS ESA 6001, Lab. de Chimie Bio-organique, UNSA*
UFR Sciences, BP 71, 06 108 NICE Cedex 2, France

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Abstract—Caulerpenyne (CYN) contents was measured in two Chlorophyceae algae, *Caulerpa taxifolia* and *Caulerpa racemosa*, between July 1999 and July 2000. Sampling was performed at three stations exhibiting increasing levels of competition with the seagrass *Posidonia oceanica*. Significant differences were observed as a function of the *Caulerpa* species, the season, and the level of competition. CYN concentrations were always greater in *C. taxifolia*, regardless of either season or level of competition (35–80 times greater, according to the season). For a given species, maximum concentrations were recorded in autumn (September/November) and minimum values occurred in spring (April/May). CYN contents decreased with increasing level of competition, whereas frond length increased over this same gradient. It would appear that when the algae are in competition with *P. oceanica*, *Caulerpa* is more inclined to accelerate vegetative growth (competition for light) than to produce secondary metabolites.

Key Words—*Posidonia oceanica*, *Caulerpa taxifolia*, *Caulerpa racemosa*, competition, caulerpenyne.

INTRODUCTION

The genus *Caulerpa*, which belongs to the Class Ulvophyceae, is made up of approximately 1000 species, most of which are found in temperate and tropical waters. *Caulerpa taxifolia* (Vahl) C. Agardh and *Caulerpa racemosa* (Forsskål) J. Agardh are currently spreading throughout the Mediterranean. *C. racemosa* is generally considered to be a Lessepsian immigrant, as are *C. scalpelliformis* and *C. mexicana* (Boudouresque et al., 1996; Piazzi et al., 1997). Conversely, the

*To whom correspondence should be addressed. E-mail: dumay@univ-corse.fr

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presence of *C. taxifolia* within the Mediterranean is most probably due to the accidental release of this alga from a public aquarium. It was observed for the first time in Monaco in 1984 (Meinesz and Hesse, 1991). Its distribution, which was on the order of 1 m^2 when initially discovered and 3 ha in 1990 (Meinesz et al., 1993), was estimated to be between 8000 and 10,000 ha in 2000 (Gravez et al., 2000) with six countries being affected (Monaco, France, Spain, Italy, Croatia, and Tunisia). *C. racemosa* has also spread rapidly within the Mediterranean. Since it was first discovered in the western basin (Livorno, Italy), the area that this alga covers has increased from 3000 m^2 in 1993 to over 300 ha in 1997 (Piazzi et al., 1997), which represents a 1000-fold increase.

Among the many secondary metabolites produced by algae belonging to the genus *Caulerpa*, caulerpenyne (CYN) is the most abundant cytotoxic sesquiterpenoid to be released. In *Caulerpa taxifolia* (Vahl) C. Agardh, this molecule makes up 0.2% (in spring) to 13% (in summer) of the alga's frond wet weight (Amade et al., 1994), values that are much higher than those observed in other *Caulerpa* species (Guerriero et al., 1994). In *C. taxifolia* and *C. racemosa* (Forsskål) J. Agardh, CYN, thought to be the most active substance, is involved either in the chemical defense of the plant against herbivore pressure or within the framework of interspecific competition (antifeedant and antifouling effects) (Lemée et al., 1993, 1997).

A variety of toxic effects due to CYN have been demonstrated in several organisms at different stages of their growth. The most harmful effect is on sea urchin eggs, which are killed by CYN (Lemée et al., 1993). This molecule, therefore, represents a potential danger to organisms living in proximity to either of these two *Caulerpa* species. The *C. taxifolia* strain from the Mediterranean contains higher CYN concentrations than the tropical strain (Guerriero et al., 1992). In addition, it exhibits exceptional growth in the Mediterranean in terms of both its population densities and the length of its fronds (Meinesz and Hesse, 1991).

The phases of vegetative growth in *P. oceanica* and *C. taxifolia* are reversed (Boudouresque et al., 1995; De Villèle and Verlaque, 1995). Thus, the leaves of *P. oceanica* are larger in winter and spring than the fronds of *C. taxifolia*, the period during which the growth of the latter species is at a minimum (Meinesz et al., 1993, 1995). Conversely, it is in summer and autumn that fronds of this alga reach their maximum length of 65 cm (see Meinesz and Hesse, 1991). During this period, the biomass of *C. taxifolia* can exceed 10 kg wet weight/m^2 (Meinesz and Hesse, 1991; De Villèle and Verlaque, 1995).

The aim of the present study was to assess the interspecific competition between either *C. taxifolia* or *C. racemosa* and the seagrass *Posidonia oceanica* (L.) Delile. This impact was assessed by quantifying the levels of CYN in the two *Caulerpa* species under different conditions of competition. *P. oceanica* is a key species in Mediterranean coastal ecosystems (Pergent, 1992), and regressions of

these seagrass beds are not without consequence, both from an ecological and economic point of view. Although a certain number of studies have quantified the impact of *C. taxifolia* on *P. oceanica* seagrass beds (Verlaque and Fritayre, 1994; De Villèle and Verlaque, 1995; Boudouresque et al., 1995; Francour et al., 1995), the same is not true of the impact of *P. oceanica* on these two *Caulerpa* species (Ceccherelli et al., 2000).

METHODS AND MATERIALS

Sampling of *C. taxifolia* was performed every two months, from July 1999 to July 2000, at the Cap Martin site (Alpes Maritime, France), at a depth of 10.0 m, and at the Antignano site (Livorno, Italy) for *C. racemosa*, at a depth of 2.0 m (Figure 1). For each site, three levels of competition were considered (Figure 2): **Station 0**—absence of *P. oceanica*; **Station 1**—simple contact between *P. oceanica* and either *C. taxifolia* or *C. racemosa* (boundaries), in which the fronds of *Caulerpa* just touch the sheath and leaves of *P. oceanica*; and **Station 2**—strong interaction between *P. oceanica* and either *C. taxifolia* or *C. racemosa* (mixed population), in which the fronds of *Caulerpa* penetrate inside foliar shoots and rhizomes, between sheaths and leaves of *P. oceanica*.

The sampling stations at each site were within the same meadow and exhibited similar biological/physical and chemical characteristics (shoot density, sediment,

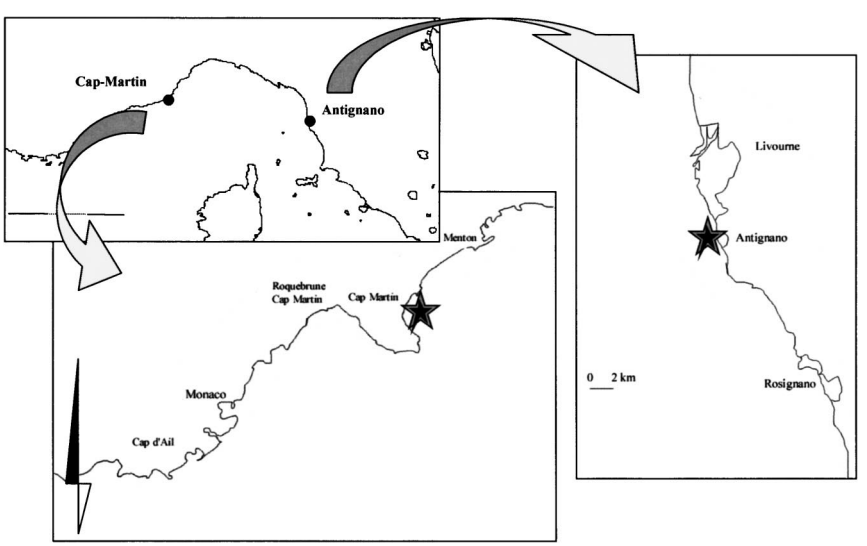


FIG. 1. Location of the sampling sites: Roquebrune-Cap Martin (France) for *Caulerpa taxifolia* and Antignano (Livorno, Italy) for *Caulerpa racemosa*.

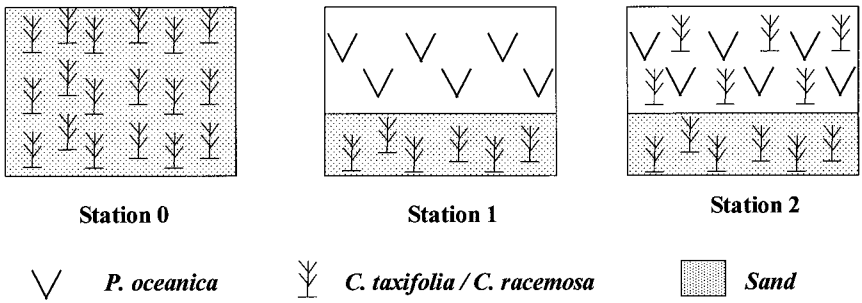


FIG. 2. Diagrams representing the three levels of competition between *P. oceanica* and the two *Caulerpa* species.

depth, temperature, salinity, hydrodynamics, etc.). The only difference between stations involved the presence and/or density of *C. taxifolia*.

Five samples were taken from each station. The algae were subsequently processed according to the protocol described by Amade et al. (1996). Briefly, they were rinsed in fresh water and stored at –20°C in methanol at a concentration of 5 g wet weight of *Caulerpa* in 50 ml of MeOH (MeOH, 95–98% Chromanorm; HPLC quality) in order to avoid any degradation of the CYN.

Extraction of CYN was performed directly in MeOH. To ensure the total diffusion of the CYN present within the syncytium, samples were sonicated for approximately 5 min. CYN measurements were performed using high performance liquid chromatography (Amade et al., 1996). Thus, 10 μl of sample was injected into a glass column of 5 μm silica (100 $\times$ 3 mm, Chrompack) and eluted with a MeOH–water solvent mixture (8:2) at a speed of 0.5 ml/min. As the retention time of CYN is on the order of 2.8 min, the injection time for each sample was set at 6 min. Measurements were performed at a UV wavelength of 254 nm, sensitivity = 0.32. The HPLC pump (Waters 600), equipped with an automatic injector (Waters 717), was monitored by specific software (Millennium Waters software) that also controlled the PAD data acquisition (Photodiode Array Detector Waters 996). This system allowed CYN peaks to be identified during elution, both in real time and under the measurement conditions. Three replicates were performed for each sample to assess the analytical dispersion. The standard curve, drawn up by using purified CYN, allowed the determination of CYN levels in the samples (Amade et al., 1994). A direct relationship between HPLC peaks and CYN levels was thus obtained.

Once normality (Shapiro and Wilks test) and variance homogeneity (Bartlett test) were tested, a one-way ANOVA was performed (as there exists a positive relationship between season and station) coupled with a Tukey multiple comparisons test in order to compare frond length. When the data were not normal (e.g., CYN levels), a nonparametric Kruskal-Wallis test was used coupled with a

Neuman-Keuls-Shapiro test in order to detect differences (Zar, 1984). The software Statgraphics Plus (v. 2.1) for Windows was used.

RESULTS

Variations in Caulerpenyne Content. CYN concentrations varied according to season (Kruskal-Wallis test; $P < 0.01$). For *C. taxifolia* (Figure 3), a maximum CYN production was observed in fall (September), followed by a decrease to reach minimum values in spring (March/May), and this regardless of the level of competition.

For a given season, CYN concentrations also varied as a function of competition (Kruskal-Wallis test; $P < 0.01$), and a decrease in CYN was observed with increasing levels of competition. In July, for example, station 0 exhibited values of 3.70 ± 0.68 mg CYN/g wet wt, whereas values were on the order of 1.99 ± 0.75 mg CYN/g wet wt and 1.42 ± 0.57 mg CYN/g wet wt for stations 1 and 2, respectively (Figure 3).

The severe storms that hit western Europe in December of 1999 caused exceptional hydrodynamic conditions on the Ligurian and Tuscan continental shelves. This phenomenon brought with it the erosion of 3–5 cm of substrate, as was clearly seen by the laying bare of the matte in the *P. oceanica* beds. In light of its shallow root system (rhizoids of 0.5–1 cm in length), *C. racemosa* was uprooted and a drastic reduction in its bottom cover was observed throughout the sampling site. Only a few individuals present within the rhizomes of *P. oceanica* escaped: the study of competition at this site was, therefore, stopped prematurely. Nevertheless, as was observed for *C. taxifolia*, the trend was toward a decrease in CYN levels with

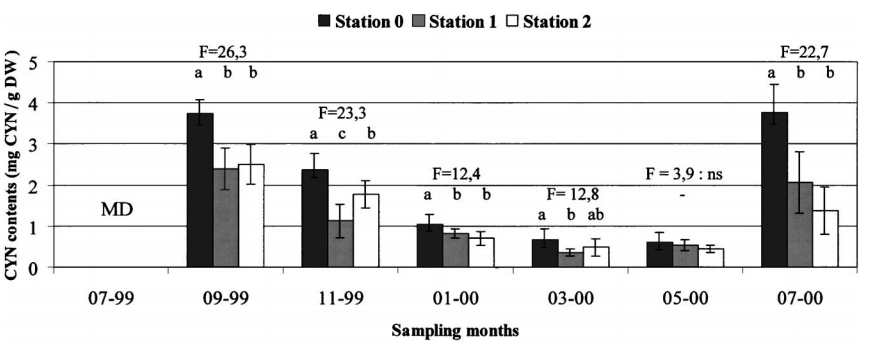


FIG. 3. CYN concentrations in *C. taxifolia* as a function of both season (fronds and stolons combined) and level of competition. No data are available for July 1999 (MD: missing data). Also shown are the ANOVA (or Kruskal-Wallis) test results (ns: nonsignificant). Tukey (or NKS) test is presented when ANOVA (or Kruskal-Wallis) test is significant. Values that do not differ at the 0.01 level are noted with the same letter.

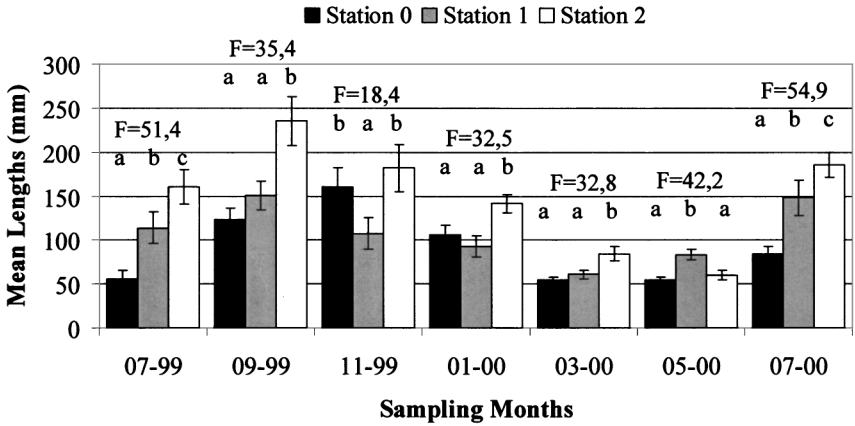


FIG. 4. Mean frond lengths for *C. taxifolia* as a function of both season and level of competition. Also shown are the ANOVA (or Kruskal-Wallis) test results (ns: nonsignificant). Tukey (or NKS) test is presented when ANOVA (or Kruskal-Wallis) test is significant. Values that do not differ at the 0.01 level are noted with the same letter.

increasing levels of competition. Indeed, in October 1999, CYN concentrations went from 0.07 ± 0.010 mg CYN/g wet wt for the station without competition to 0.03 ± 0.006 mg CYN/g wet wt for the station with a high level of competition, which represents a decrease of 42%. The station presenting intermediate competition levels generated values of 0.06 ± 0.01 mg CYN/g wet wt.

The mean CYN values were much higher in *C. taxifolia* than in *C. racemosa*. For example, in November 1999 values at the stations presenting no competition were on the order of 2.37 ± 0.41 mg CYN/g wet wt and 0.03 ± 0.014 mg CYN/g wet wt, respectively. There was, therefore, 80 times more CYN in *C. taxifolia*.

Variation in Frond Length. The frond length in *C. taxifolia* varied as a function of season (Figure 4), with a maximum autumn value (September–November) and a minimum in spring (March–May) (one-way ANOVA, $P < 0.01$). The longest fronds (one-way ANOVA; $P < 0.01$) were those in close contact with the *P. oceanica* seagrass beds (maximum level of competition). Thus, in July 2000, the algae in contact with *P. oceanica* exhibited mean frond lengths of 185.5 ± 13.9 mm vs 84.5 ± 8.1 mm where no such competition occurred; the station presenting moderate competition levels exhibited an intermediate value of 148.1 ± 20.3 mm.

Similar results were recorded for *C. racemosa*, with frond length also varying as a function of season (maximum length in November) and the largest fronds being those in the closest contact with *P. oceanica* (Figure 5). For example, in October 1999, fronds in close contact with *P. oceanica* exhibited a mean length of 19.13 ± 2.64 mm, followed by a value of 17.19 ± 1.82 mm for moderate contact, and finally 14.94 ± 1.85 mm in the absence of interspecific competition.

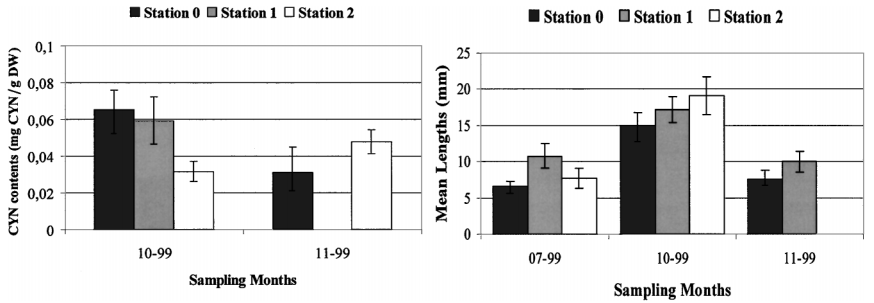


FIG. 5. CYN concentrations and mean frond lengths in *C. racemosa* as a function of both season (fronds and stolons combined) and level of competition.

DISCUSSION

The seasonal variations in CYN concentrations observed in *C. taxifolia* confirm previous results (Amade et al., 1996). As pointed out by Lemée et al. (1997), these variations help to explain the maximum and minimum toxicities observed for *C. taxifolia* in summer/fall and in spring, respectively.

Interspecific competition also appears to affect CYN levels, with those individuals experiencing maximum competition levels presenting the lowest CYN concentrations. Moreover, individuals under conditions of intense competition exhibited longer maximum frond lengths, which is a classical phenomenon in plants, termed phototropism.

Although these trends were less evident than in *C. taxifolia*, there appears to be a decrease in CYN levels in *C. racemosa* between October and November. Further studies over a longer time period will need to be undertaken to confirm these results. In addition, there was a great difference in CYN concentrations between the two algae. Values differed by a factor of 35–80, depending on the season, for a given level of competition.

We detected a variation in the length of the fronds in *C. taxifolia* throughout the year 1999/2000, with maximum lengths in early autumn and a minimum in spring. This evolution is similar to that observed in *P. oceanica*, but presents a lag period of approximately three months. Indeed, leaf lengths in *Posidonia oceanica* exhibited maximum values in June–July and minimum values in winter (Wittman, 1984; Pergent and Pergent-Martini, 1988). This tends to confirm the hypothesis of competition for light between these two photoautotrophs; growth of the *Caulerpa taxifolia* fronds corresponds to the plant's response in the acquisition of this resource. Studies involving another invasive species, namely *Sargassum muticum*, have revealed that the indigenous algal community is altered as a result of, or in response to, an interspecific competition for both nutrients and light

(Stæhr et al., 2000.). It, therefore, appears that this phenomenon often occurs when several species compete for the same ecological niche.

Variations in the seasonal growth of *Caulerpa taxifolia* appear to be widespread within the Mediterranean. Such variations have also been reported by Meinesz et al. (1993) and Belsher et al. (1994), who described minimum growth in winter and spring and maximum values in summer and autumn. It seems that such seasonal variation also exist in the frond lengths of *C. racemosa* (Piazzi et al., 1997).

In summary, this study shows that CYN concentrations in *C. taxifolia* exhibit seasonal variations with maximum values in autumn and minimum values in spring. A similar trend is observed for mean frond lengths. There appears to be a direct relationship between frond length and CYN concentration. Althouth a number of parameters may be implicated, the most likely reason for these significant physiological modification in *C. taxifolio* is the presence of *P. oceanica.* Results suggest that the presence of this phanerogam leads to a decrease in CYN production with a concomitant increase in frond length. Similar observations were made for *C. racemosa*, although these last results need confirmation.

The decrease in CYN concentrations, with the concomitant increase in frond length, under conditions of competition with *Posidonia oceanica* could be the result of a modified metabolism in the alga. The increased energy allocated to growth of fronds (nutrient resources, light) would be at the cost of another function (e.g., production of secondary metabolites). If this hypothesis were to be confirmed, the production of secondary metabolites, thought to intervene under conditions of competition, would not be the strategy adopted by *C. taxifolia* when competing with *Posidonia oceanica*. Instead, this alga would appear to use an "exploitation"-type (e.g., use of light) rather than an "interference"-type competitive strategy (e.g., synthesis of active molecules) (Barbault, 1997; Ramade, 1990).

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MYCORRHIZAL COLONIZATION AND PLANT GROWTH AFFECTED BY AQUEOUS EXTRACT OF *Artemisia princeps* var. *orientalis* AND TWO PHENOLIC COMPOUNDS

K. W. YUN* and S. K. CHOI

Department of Oriental Medicine Resources
Sunchon National University
Suncheon 540-742, Republic of Korea

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Abstract—The effects of an aqueous extract of *Artemisia princeps* var. *orientalis* and two phenolic compounds on mycorrhizal colonization and plant growth have been investigated. Greenhouse studies showed that the inhibitory effect of the extract on mycorrhizal colonization and plant growth increased in proportion to the concentration of the extract. When the mycorrhizal test plants were treated with an increasing concentration of phenolic compounds, the mycorrhizal colonization in roots of the test plant and the plant growth were decreased. There were strong indications that mycorrhizal fungi mitigated the inhibitory influence of shoot extract of *A. princeps* var. *orientalis* and phenolic compounds.

Key Words—*Artemisia princeps* var. *orientalis*, aqueous extract, caffeic acid, *p*-coumaric acid, mycorrhizal fungi, plant growth.

INTRODUCTION

It has been reported that *Artemisia princeps* var. *orientalis* exhibits allelopathic effects via aqueous extracts (Kil and Yun, 1992), residues (Yun and Kil, 1992), and volatile constituents (Yun et al., 1993). Yun (1991) identified benzoic acid and 16 phenolic compounds as water-soluble allelochemicals of *A. princeps* var. *orientalis*.

Phenolic substances have been identified as allelopathic inhibitors more often than any other class of chemicals. They are readily soluble in water and easily leached from plant tissue (Horsley, 1977). Some have been reported to interfere with ontogenic processes, energy transfer processes, and nutrient uptake

*To whom correspondence should be addressed. E-mail: ykw@sunchon.sunchon.ac.kr

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or transport. Corcoran et al. (1972) found that several tannins, benzoic acid, and cinnamic acid inhibit gibberellic acid-induced growth. Einhellig and Kuan (1971) showed that scopoletin inhibits photosynthesis in tobacco. Caffeic acid and *p*-coumaric acid are widely known as allelochemicals (Rice, 1984). There has been a report that leaf area expansion of cucumber seedlings is inhibited by ferulic and *p*-coumaric acid, depending on the concentration and the reduction in leaf expansion because of reduced hydrostatic pressure for cell expansion (Blum et al., 1985).

Plant–mycorrhizal fungus association enhances uptake and transport of soil nutrients to the plant via fungus (Cox et al., 1975; Allen, 1991). Siqueira et al. (1991) suggested that declines in arbuscular mycorrhizal (AM) fungal populations in the field can negatively affect the growth of crop plants. Tinker (1978) reported that vesicular arbuscular mycorrhizae aid host plants in the acquisition of phosphorus and certain micronutrients. However, allelochemicals can also adversely affect the normal functioning of mycorrhizal fungi associated with their root systems (Olsen et al., 1971; Theodorou and Bowen, 1971), thus reducing the beneficial effects of AM fungi.

The purpose of the present study is to examine the effect of an aqueous extract of *A. princeps* var. *orientalis* and two phenolic compounds on mycorrhizal colonization and plant growth of three plant species.

METHODS AND MATERIALS

Preparation of Aqueous Extract. Aqueous extract was prepared as follows; 100 g of dry shoots of *A. princeps* var. *orientalis* were soaked in 1 liter of distilled water at 20°C. After 24 hr, it was filtered through a sieve and the filtrate was considered as 100% extract. The extract was then diluted to reduce concentration to 50% and 10% with distilled water.

Mycorrhizal Fungal Materials. Seeds of three test plants, *Digitaria sanguinalis*, *Plantago asiatica*, and *Oenothera odorata*, were germinated in Petri dishes, and the seedlings transplanted into pots filled with plant-growth substrate (vermiculite–perlite–autoclaved soil 8:2:1). When the plants were 2 months old, they were inoculated with mycorrhizal fungi according to the procedure of Little and Maun (1996). The procedure consisted of growing corn seedlings in soil containing mycorrhizal fungi. When the seedlings were 2 months old, the presence of mycorrhizal fungi was ascertained. The corn roots with mycorrhizal fungi were washed, weighed into 5-g batches, and placed at 5-cm depth in new plastic pots (Yun and Maun, 1997).

Phenolic Compounds. Gas chromatography was used to identify and analyze the water-extractable phytotoxic substances of *A. princeps* var. *orientalis*. Benzoic, phenylacetic, salicylic, *t*-cinnamic, *p*-hydroxybenzoic, vanillic, gentisic, *o*-hydroxycinnamic, protocatechuic, syringic, *p*-coumaric, ferulic, and caffeic acids

and *m*-hydroxybenzone, catechol, phloroglucinol, *p*-phenylbenzaldehyde were detected in the leaf aqueous extract of *A. princeps* var. *orientalis* (Yun, 1991). Among these acids, caffeic acid and *p*-coumaric acid were chosen for the study, because these two phenolic acids were consistently detected in *A. princeps* var. *orientalis* extracts. Chemicals were purchased from Sigma Co. (St. Louis, Missouri; USA). Each acid was diluted to 5×10^{-5}, 5×10^{-4}, and 5×10^{-3} M.

Effect of Aqueous Extract on Mycorrhizal Fungal Colonization and Plant Growth. The three test plants, *Digitaria sanguinalis, Plantago asiatica*, and *Oenothera odorata*, are representative weed species in South Korea and did not occurr with *A. princeps* var. *orientalis*. They were inoculated with mycorrhizal fungi and placed in the greenhouse maintained at 25°C during the day and 18°C at night. Plants were allowed to grow for an additional two months in order to ensure that their roots had been colonized by mycorrhizal fungi. The plants were then exposed to the aqueous extract treatments, which consisted of the addition of 40 ml of 10, 50, and 100% shoot extracts of *A. princeps* var. *orientalis* to the soil in each pot. Extracts were added every two days for one month. Distilled water was used for the control plants. Six replications were used.

After 30 days, the experiment was terminated. Plants were taken out of the pots and washed carefully to remove plant-growth substrate from roots. Shoot and root lengths and dry weight were recorded. A 2 g sample of the root of the test plants was taken from each plant to determine mycorrhizal colonization. The root samples were stained in Chlorazol Black-E stain (Brundrett et al., 1984). Percent colonization of the stained root was determined by the magnified-intersection method (McGonigle et al., 1990).

Effect of Acids on Mycorrhizal Fungal Colonization and Plant Growth. The same experimental procedure as described above was followed, except that the two phenolic compounds, caffeic acid and *p*-coumaric acid, instead of the aqueous extract were used. The test plant was *Digitaria sanguinalis*.

RESULTS

The Effects of Aqueous Extract of Artemisia princeps var. orientalis on Mycorrhizal Colonization and Plant Growth. The effect of the shoot extract of *A. princeps* var. *orientalis* on mycorrhizal fungal colonization in the root of the three test plants is shown in Table 1. In *Digitaria sanguinalis*, arbuscular vesicular mycorrhizal (AM) colonization decreased with an increase in the concentration of aqueous extract. In *Digitaria sanguinalis*, the uncolonized area and hyphal colonization were not significantly altered by the three concentrations. However, the percent vesicular colonization decreased significantly, with an increase in the percent concentration of the aqueous extract. *Plantago asiatica* was more susceptible to the extracts. The uncolonized area increased significantly, and

TABLE 1. COLONIZATION OF ROOTS BY MYCORRHIZAL FUNGI FOR *Digitaria sanguinalis*, *Plantago asiatica* AND *Oenothera odorata* PLANTS GROWN IN POTS UNDER GREENHOUSE CONDITIONS AND EXPOSED TO 0, 10, 50, AND 100% CONCENTRATIONS OF *Artemisia princeps* var. *orientalis* SHOOT EXTRACTS[a]

	Colonization (%, mean ± SE)								
	Digitaria sanguinalis			*Plantago asiatica*		*Oenothera odorata*			
Concentration (%)	UNC	Vesicle	Hyphae	UNC	Vesicle	UNC	Vesicle	Hyphae	Arbuscule
Nonmycorrhizal[b]									
0	93.0 ± 0.4a	2.0 ± 0.0b	4.1 ± 0.1b	77.1 ± 4.8a	22.9 ± 4.8b	85.6 ± 1.2a	1.2 ± 0.8b	12.7 ± 2.4b	0.65 ± 0.07c
Mycorrhizal									
0	52.6 ± 4.7b	19.2 ± 3.6a	26.6 ± 2.5a	42.1 ± 8.6b	57.2 ± 9.6a	52.7 ± 5.5c	12.3 ± 5.4ab	19.8 ± 3.1a	15.3 ± 2.20ab
10	65.4 ± 6.3b	7.8 ± 2.1ab	26.8 ± 2.3a	53.5 ± 7.5b	45.9 ± 7.8a	62.9 ± 4.2bc	11.2 ± 2.6ab	8.4 ± 2.0bc	18.2 ± 2.10a
50	69.6 ± 6.0b	1.0 ± 0.7b	29.4 ± 5.2a	59.2 ± 5.5ab	40.9 ± 5.5ab	67.8 ± 3.0b	17.5 ± 1.4a	6.9 ± 1.2bc	17.8 ± 0.20bc
100	67.8 ± 2.8b	0.0 ± 0.0b	32.7 ± 2.8a	76.8 ± 4.7a	23.2 ± 4.7b	87.5 ± 3.5a	7.2 ± 1.2ab	3.9 ± 0.9c	11.4 ± 0.01c

[a] Percent colonization by vesicles, hyphae and arbuscules and percent uncolonized (UNC) roots were determined according to the method of McGonigle et al. (1990). Values in a column followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.
[b] Nonmycorrhizal means no-inoculation of plants by mycorrhizal fungi.

vesicular mycorrhizal colonization of the root was inhibited significantly by the extract. In the case of *Oenothera odorata*, vesicular, hyphal, and arbuscular mycorrhizal colonizations were significantly inhibited by the extract, and the inhibition was concentration-dependent.

The shoot and root elongations of the three test plants treated with aqueous extract of shoots of *A. princeps* var. *orientalis* are shown in Table 2. Shoot and root elongations of *Digitaria sanguinalis* were inversely proportional to the extract concentrations especially at the 50 and 100% concentrations. Mycorrhizal fungi mitigated the injurious effects of aqueous extracts on the elongation of shoots at all three concentrations and elongation of roots at 50 and 100% concentrations. The shoot and root elongation of *Plantago asiatica* was also influenced by different concentrations of aqueous extracts. A significant decrease in shoot elongation occurred at 50 and 100% concentrations, while root elongation decreased significantly from control only at 100% concentration of extract. In the case of *Oenothera odorata*, shoot elongation was significantly higher in the 100% extract compared with nonmycorrhizal control. None of the other treatments differed significantly. The root elongation decreased significantly at 50 and 100% aqueous extracts. The colonization of roots by AM fungi was beneficial to both *P. asiatica* and *O. odorata* for decreasing the allelopathic impact of *A. princeps* var. *orientalis*. Mycorrhizal fungi association significantly increased the dry weight of all three species compared with the nonmycorrhizal control. Mycorrhizal plants exposed to the three concentrations of aqueous extract were not significantly different in their dry weight from the nonmycorrhizal control, suggesting that mycorrhizal fungi did mitigate the harmful effects of allelopathy. However, the beneficial effect of AM fungi seen in the mycorrhizal control was lost.

Effects of Phenolic Compounds on Mycorrhizal Colonization and Plant Growth. Percent root colonization of *Digitaria sanguinalis* by mycorrhizal was inhibited by the two phenolic compounds, caffeic acid and *p*-coumaric acid (Table 3). The uncolonized area of nonmycorrhizal plants exposed to caffeic and *p*-coumaric acids was significantly higher than mycorrhizal plants. As the concentration of these two acids was increased, there was an increase in percent uncolonized area. Similar results were also observed for colonization by mycorrhizal hyphae. *Digitaria sanguinalis* treated with caffeic acid and *p*-coumaric acid had the lowest mycorrhizal colonization when the highest concentration (5×10^{-3} M) of each acid was used. Percent colonization by vesicles showed an increase at intermediate concentrations of caffeic acid. However, at the highest concentration, vesicular colonization was not significantly different from control (Table 3). Inoculation of *D. sanguinalis* plants by mycorrhizal fungi increased shoot elongation and dry weight compared to nonmycorrhizal controls (Table 4). However, root elongation was not altered. Exposure of plants to *p*-coumaric acid did not show a clear-cut effect on shoot elongation. There was no change in shoot elongation at 5×10^{-5} M concentration but as the concentration increased there was a slight decline. The

TABLE 2. SHOOT ELONGATION, ROOT ELONGATION, AND DRY WEIGHT OF TEST SPECIES PLANTS GROWN IN POTS IN A GREENHOUSE[a]

Species	Concentration (%)				
	Nonmycorrhizal, 0	Mycorrhizal			
		0	10	50	100
Shoot elongation (cm)					
Digitaria sanguinalis	13.7 ± 0.3b	29.3 ± 2.5a	21.0 ± 3.0b	21.3 ± 3.4b	16.4 ± 3.8b
Plantago asiatica	11.0 ± 0.5a	11.6 ± 0.5a	10.2 ± 0.7a	7.2 ± 0.6b	7.4 ± 0.4b
Oenothera odorata	6.7 ± 0.4b	7.7 ± 0.3ab	7.4 ± 0.3ab	7.7 ± 0.2ab	8.2 ± 0.3a
Root elongation (cm)					
Digitaria sanguinalis	17.0 ± 0.5b	21.9 ± 2.3ab	26.0 ± 3.1a	17.4 ± 1.2b	15.7 ± 1.5b
Plantago asiatica	21.6 ± 0.6ab	24.1 ± 1.8a	22.6 ± 1.0ab	22.4 ± 0.6ab	19.7 ± 1.0b
Oenothera odorata	21.8 ± 1.2a	23.5 ± 0.9a	20.4 ± 0.4ab	17.6 ± 1.8bc	14.8 ± 1.2c
Dry weight (g)					
Digitaria sanguinalis	0.20 ± 0.05b	0.72 ± 0.04a	0.52 ± 0.08ab	0.38 ± 0.09b	0.32b ± 0.08b
Plantago asiatica	2.71 ± 0.25bc	4.58 ± 0.55a	3.34 ± 0.45b	2.26 ± 0.25bc	1.70c ± 0.17c
Oenothera odorata	1.35 ± 0.12c	2.92 ± 0.40a	1.31 ± 0.19b	1.67 ± 0.28b	1.33b ± 0.05b

[a] Values given are mean ± SE. Plants were inoculated by mycorrhizal fungi and exposed to 0, 10, 50, and 100% concentrations of *Artemisia princeps* var. *orientalis* aqueous extracts. Values in each line followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.
[b] Nonmycorrhizal means no inoculation of plants by mycorrhizal fungi.

TABLE 3. COLONIZATION OF ROOTS BY MYCORRHIZAL FUNGI FOR *Digitaria sanguinalis* PLANTS GROWN IN POTS UNDER GREENHOUSE CONDITIONS AND EXPOSED TO PHENOLIC COMPOUNDS[a]

	Colonization (%, mean ± SE)				
	Caffeic acid			*p*-Coumaric acid	
Concentration (M)	UNC	Vesicle	Hyphae	UNC	Hyphae
Nonmycorrhizal[b]					
0	83.9 ± 1.7a	0.8 ± 0.1b	15.3 ± 1.0b	77.5 ± 2.6b	22.5 ± 2.6b
Mycorrhizal					
0	51.4 ± 3.5c	2.7 ± 0.9b	45.9 ± 2.9a	54.3 ± 1.8c	45.7 ± 2.1a
5×10^{-5}	62.1 ± 5.0b	5.3 ± 2.1ab	56.3 ± 3.4a	56.3 ± 3.4c	43.7 ± 3.4a
5×10^{-4}	69.5 ± 2.5b	9.8 ± 1.6a	20.7 ± 5.9b	61.3 ± 5.9c	38.7 ± 5.9a
5×10^{-3}	90.0 ± 3.7a	2.6 ± 0.7b	7.4 ± 3.2c	89.8 ± 2.4a	1.2 ± 2.4c

[a] Percent colonization by vesicles, hyphae, and arbuscules and percent uncolonized (UNC) area of roots were determined according to the method by McGonigle et al. (1990). Values in a column followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.
[b] Nonmycorrhizal means no-inoculation of plants by mycorrhizal fungi.

TABLE 4. SHOOT ELONGATION, ROOT ELONGATION, AND DRY WEIGHT OF *Digitaria sanguinalis* GROWN IN POTS, COLONIZED BY MYCORRHIZAL FUNGI, THEN EXPOSED TO PHENOLIC COMPOUNDS

	Plant growth (mean ± SE)		
Concentration (M)	Shoot elongation (cm)	Root elongation (cm)	Dry weight (g)
Caffeic acid			
0 (NM)[a]	33.9 ± 5.3c[b]	21.8 ± 1.3a	0.43 ± 0.08c
0	48.5 ± 2.6ab	21.8 ± 2.6a	1.16 ± 0.09a
5×10^{-5}	49.7 ± 0.6a	20.1 ± 2.1a	1.01 ± 0.09a
5×10^{-4}	40.5 ± 0.1bc	24.8 ± 1.0a	0.69 ± 0.01b
5×10^{-3}	43.5 ± 1.2ab	20.7 ± 2.0a	0.70 ± 0.04b
p-Coumaric acid			
0 (NM)	35.3 ± 1.1b	22.9 ± 3.1a	0.31 ± 0.02c
0	49.4 ± 1.6a	22.9 ± 1.9a	1.18 ± 0.14a
5×10^{-5}	39.1 ± 0.7b	23.2 ± 1.8a	0.76 ± 0.10b
5×10^{-4}	37.7 ± 1.1b	19.0 ± 1.9a	0.61 ± 0.05b
5×10^{-3}	21.2 ± 3.3c	20.2 ± 1.2a	0.23 ± 0.04c

[a] NM means nonmycorrhizal.
[b] Values in a column followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

p-coumaric acid exhibited a decline in shoot elongation with an increase in concentration. The root elongation was not affected by any of the four concentrations of both caffeic and *p*-coumaric acids. The two acids had a clear negative impact on the dry weight of *D. sanguinalis* plants. As the concentrations of the two acids were increased, there was a decrease in dry weight (Table 4).

DISCUSSION

The present study demonstrates the effects of shoot extracts of *Artemisia princeps* var. *orientalis* and different concentrations of caffeic and *p*-coumaric acids on root mycorrhizal colonization, shoot and root elongations, and dry weight of the weedy plant species.

We emphase four main contributions of this study. First, as expected, the inoculation of plants by arbuscular mycorrhizal fungi increased the dry weight of all three species of weeds compared to nonmycorrhizal controls. Several studies show that mycorrhizal fungi provide additional nutrients, especially phosphorus to the host (Allen, 1991). The fungal hyphae make connections with the root hair and colonize a greater volume of soil than the roots of nonmycorrhizal plants. Second, the shoot extracts of *A. princeps* var. *orientalis* and phenolic compounds were clearly inhibitory to the dry weight per plant of all three species. Kil and Yun (1992) reported similar inhibitory effects of the aqueous extract of *A. princeps* var. *orientalis* on seed germination and seedling growth of six plant species. The only difference was that in this study, mycorrhizal inoculated plants were treated with the aqueous extracts, while Kil and Yun (1992) applied the extracts to nonmycorrhizal plants. In this study, the extract of *A. princeps* var. *orientalis* inhibited mycorrhizal fungal colonization in *Plantago asiatica* and contributed to a decline in plant growth. Similarly, although mycorrhizal colonization of *Oenothera odorata* was inhibited by the extract, the shoot elongation of the plant was not affected. The degree of inhibition effect of the extract on mycorrhizal plants is smaller than that on nonmycorrhizal plants. This is similar to the first study, clearly demonstrating the effect of allelopathic interference on the function of mycorrhizae (Nilsson et al., 1993). Caffeic acid showed a stimulatory effect on hyphal mycorrhizal colonization at 5×10^{-5} M, but it produced an inhibitory effect at 5×10^{-4} and 5×10^{-3} M. The inhibition of hyphal mycorrhizal colonization by *p*-coumaric acid was proportional to the concentration. Wacker et al. (1990) found that ferulic acid led to an inhibition of mycorrhizal colonization of asparagus by *Glomus fasciculatum*, and it was dependent upon the concentration. Kil et al. (1989) reported that *p*-coumaric acid showed an inhibitory effect on seed germination and nonmycorrhizal seedling growth of *Lactuca sativa* at concentrations of 5×10^{-5}, 5×10^{-4}, and 5×10^{-3} M. Seedling growth of mycorrhizal plants was less inhibited than that of nonmycorrhizal plants by *Empetrum* extract (Nilsson et al., 1993). Nilsson et al.

(1993) proposed that the spread of mycorrhizal infection and uptake of nutrients by roots and mycorrhizae were more sensitive to the extract than seed germination and radicle growth. Pedersen et al. (1991) showed caffeic and methylenedioxycinnamic acids reduced mycorrhizal infection of *Glomus fasciculatum* in asparagus and that it could influence the decline of plant growth. Third, the beneficial effects of colonization by mycorrhizal fungi were lost when mycorrhizal plants were treated with shoot extracts of *A. princeps* var. *orientalis* and higher concentrations of caffeic and *p*-coumaric acids. Siqueira et al. (1991) suggested that VAM fungal inoculation reduced herbicide-induced leaf injury and alleviated the phytotoxic effects. Herbicide effects were dependent on soil-applied concentrations and the presence of VAM, and VAM effects were dependent on the kinds of herbicide and the concentration of the herbicides. Finally, we emphasize the mitigating effects of mycorrhizal fungi on the deleterious effects of allelopathy. A plant colonized by AM fungi was better equipped to survive the inhibitory impact of extracts of allelopathic plants and phenolic acids.

The results of this study lead to the conclusion that the aqueous extract of *Artemisia princeps* var. *orientalis* and two phenolic acids inhibit mycorrhizal fungal colonization and plant growth, and the degree of inhibition of mycorrhizal colonization and plant growth by the aqueous extract and the two phenolic acids showed similarities.

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SEMIOCHEMICALS AND SOCIAL SIGNALING IN THE WILD EUROPEAN RABBIT IN AUSTRALIA: I. SCENT PROFILES OF CHIN GLAND SECRETION FROM THE FIELD

R. A. HAYES,* B. J. RICHARDSON, and S. G. WYLLIE

Centre for Biostructural and Biomolecular Research
University of Western Sydney, Hawkesbury
Locked bag 1797, Penrith South Delivery Centre
NSW 1797, Australia

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Abstract—The European rabbit (*Oryctolagus cuniculus*) uses the secretion of the chin gland in the maintenance of social status. Previous work has concentrated on secretion collected directly from the animal. In this study, the analysis was conducted by collecting scent marks made by free-ranging animals. Scent marks were found to be concentrated at the center of the area controlled by a social group, and at the boundaries between two adjacent social groups. Only the mark from dominant animals could be identified. Marks were also collected from the skin of rabbits, where they had been placed by the dominant individual. The mark found on the head of a subordinate animal may, in the future, be used to identify the dominant animal of the social group, who placed the mark.

Key Words—Rabbit, *Oryctolagus cuniculus*, social structure, glandular secretion, gas chromatography, GC-MS, behavior, olfactory communication, field studies.

INTRODUCTION

The majority of the work that has been conducted to examine the constituents of mammal scent gland secretions has been undertaken on the whole secretion. Animals have been trapped and the secretion collected directly from the site of production. There are few studies where the animals were allowed to scent mark and the mark itself collected for examination. Where such attempts have been made, the volatile components of the mark are often not the focus of the study (e.g., Singer

*To whom correspondence should be addressed. E-mail: r.hayes@uws.edu.au

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et al., 1986, 1989; Belcher et al., 1990). The difficulty with using whole secretions is that there is no guarantee that the signal that an animal receives in a field situation is composed of the entire secretion. It may be that only part of the secretion is used to pass on a message, and it follows that the signaling portion of the secretion will be obtained during studies of the volatiles from field collected scent marks.

Another difficulty with previous semiochemical studies is that limited data are available on the semiochemical profiles of free-ranging animals. Most of the animals examined have been kept in cages (e.g., Brownlee et al., 1969; Black-Cleworth and Verberne, 1975; Goodrich et al., 1990a,b; Hudson and Distel 1990; Swaisgood et al., 1999) or living in controlled environments, such as enclosures (e.g., Mykytowycz, 1958, 1979; Mykytowycz and Hesterman, 1970; Müller-Schwarze et al., 1974; Gassett et al., 1996, 1997; Kappeler, 1998; Miller et al., 1998; Burger et al., 1999). Most of these animals have been fed artificial diets. There is no guarantee that the secretions exuded by captive animals are the same as those produced by animals in the wild. In more recent years, there has been a reversal of this trend, with more studies of wild animals reported (e.g., Cantoni et al., 1996; Kazanehdari et al., 1996; Siller-Zubiri and MacDonald, 1998; Sliwa and Richardson, 1998; Bel et al., 1999; Lawson et al., 2000).

The solution to both of these problems is to collect and analyze scent marks made by members of a wild population, preferably one that is well studied and where all individuals are known and recognizable. European rabbit (*Oryctolagus cuniculus*) populations are ideal, as social groups have a relatively small home range size, strict social structure, and home range fidelity, and individuals show clear scent-marking behavior. This means that it is possible to know exactly which individuals are available to mark at a given location.

Rabbit Environmental Scent-Marking Behavior. In many species of mammals, there is a vast difference between the amount of scent mark placed at or near territorial boundaries compared with marking behavior throughout the hinterland (e.g., Johnson, 1973; Peters and Mech, 1975; Roper et al., 1993; Bel et al., 1995; Bowen and Cowan, 1980; Rosell and Nolet, 1997; Rosell et al., 1998; Sillero-Zubiri and MacDonald, 1998; Sliwa and Richardson, 1998). If scent marks are for defending territory, they should be left preferentially at territory borders (Johnson, 1973). This will create a "wall of smell" to fend off intruders, and as an individually distinctive signature of territory occupancy (Gorman and Mills, 1984; Gorman, 1990). This would, then, produce an "olfactory bowl" (Peters and Mech, 1975), with a changing odor gradient as the high concentration of marks at the boundary decreased towards the center of the territory (Bowen and Cowan, 1980). Indeed, there will most likely be a positive feedback, and the number of border marks will be even higher than would be predicted due to overmarking from adjacent groups (Sillero-Zubiri and MacDonald, 1998).

An alternative approach to this mark placement is seen in the Eurasian badger, *Meles meles*, in which, once inside the territory, marking density is inversely related

to distance from the main set. In this case, an incoming intruder that has passed the territory boundary will be progressively more likely to meet a scent mark, and, thus, become aware of territorial ownership (Roper et al., 1993). The rabbit could adopt either of these two approaches.

Previous studies on rabbit chin gland secretion have used whole secretion for the analysis, either from swabs of the chin region of live rabbits (Hayes et al., 2001a,b) or from excised glands (Goodrich and Mykytowycz, 1972). As stated above, this may be different from a scent mark deliberately placed by a rabbit and utilized by the rabbit population in the field. Rabbits scent mark objects throughout their territory (Mykytowycz and Gambale, 1969) and have been shown to mark any novel object placed into their environment (Mykytowycz, 1967, 1968, 1972; Johnson, 1973). Mykytowycz (1965) said that the marking behavior of rabbits probably saturates their territory with smell. It has been suggested that different classes of rabbits mark differentially at a particular location. Dominant males mark the entire territory of the social group but appear to concentrate on the boundaries with neighboring groups (Mykytowycz, 1968). In addition, a male is much more likely than a female to apply a scent mark to a latrine site (Mykytowycz and Gambale, 1969).

In the breeding season, much of the time spent above ground by female rabbits is on, or near, the warren (Bell, 1980). Lockley (1961) found that females seldom move more than 15 m from the warren entrance, usually much less. (There is, however, some question as to the validity of this result, and the small size of this distance may have been an artifact of the experimental design.) When young are present in a burrow, the mother will seal up the entrance, then urinate, defecate, and chin-mark the opening (Mykytowycz, 1968). Thus, females are thought to concentrate their scent marking behavior at the entrances of holes where their young are located (Gibb et al., 1978). It has, therefore, been suggested that the scent marks found throughout the territory of a rabbit social group will not be uniform, but that there will be structuring of these marks, in relation to environmental cues, such as the position of warrens and other social groups.

Scent Marking on Other Rabbits. The dominant male in a social group will scent mark the other individuals in his social group. He does this with both urine and chin gland secretion (Mykytowycz, 1968; Bell, 1980). The dominant male has been observed to chin-mark juveniles and females, but no chin-marking of subordinate males has ever been observed (Bell, personal communication). By swabbing regions of an animal other than the chin, it is proposed that a scent mark different from that seen on a swab of the chin region of the donor individual can be obtained. It is also predicted that there will be no mark on the head of the dominant male, as no other animal can or will chin-mark him.

The present study sought to examine the chin gland secretion of the wild rabbit by collecting it on “chinning poles” located throughout the field site. Differences among the marks on these poles were examined by comparing chromatograms

produced. It was hypothesized that there would be structuring in the locations and content of these marks, with marks from different classes of rabbits being found in different places within the territory. In addition, swabs were collected from the tops of the heads of the rabbits and compared to the swab from the chin and to the secretion of other animals in the social group.

METHODS AND MATERIALS

Environmental Marking. The wild population of rabbits (*Oryctolagus cuniculus*) used for this study were located at Hope Farm, Cattai National Park (150°52′E, 33°33′S), in the northwest of Sydney, Australia. This population consists of about 150 adult animals, and up to about 300 juveniles, in an area of approximately 20 ha.

To examine the scent mark itself, as opposed to secretion manually expressed from the chin gland, chinning poles were placed in the field site so that wild individuals could mark them. A series of 149 tomato stakes (90 cm) were located in lines throughout the site. The chinning poles were placed at 1 m intervals across the area in lines that crossed at least one boundary between adjacent social groups. These social group boundaries were determined by examining the location of scratch marks and by behavioral observations (such as displacement behavior, chasing, etc.). Some pole lines also extended beyond the territory of an individual social group and into communal feeding areas. The pole lines were assigned a letter from A to K, and the poles within each line given a number from 1 to 26 (Figure 1). In addition, 25 chinning poles were placed at all the active entrances of seven of the warrens within the sampling area. Each warren pole was indicated by the warren number and a letter from a to e (Figure 1). A total of 174 chinning poles were, therefore, located on the site.

A double layer of aluminum foil was placed around each pole at two heights, 10 and 70 cm from the ground. The underlayer of foil prevented contact between the top layer and the stake. The top, sample layer could, thus, be removed for analysis, while the underlayer remained. The sample layers of foil at 10 cm were considered to be experimental, as this is well within the height that an adult rabbit can easily reach to scent mark. The sample layers at 70 cm are too high to be reached by a rabbit in normal activity and were used as controls. The foil layers at these two heights experienced the same environmental conditions, but layers at 70 cm could not be scent marked by a rabbit. Although aluminum foil is not a natural substrate, and the interaction of the substrate with the secretion may differ from that seen with more usual surfaces, rabbit chin secretion is placed on many different substrates by wild rabbits, from sticks and grass to stones and piles of feces (Mykytowycz, 1968). It was assumed that aluminum foil would simulate a valid substrate for the current analysis.

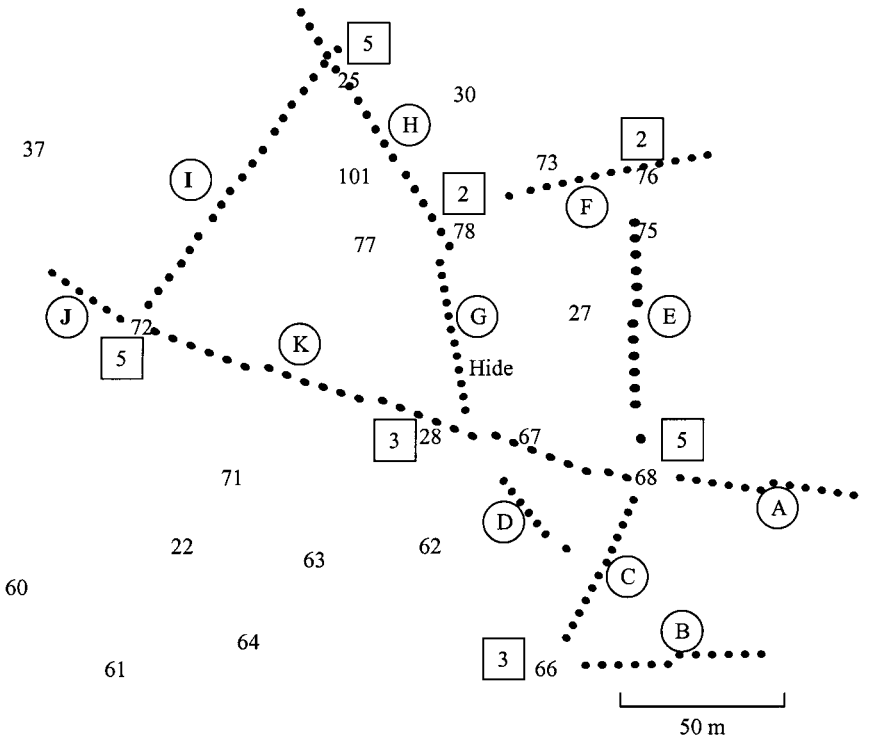


FIG. 1. A map of the central portion of the Cattai field site where the chinning poles were located. Numbers indicate warrens where trapping occurred. Chinning pole lines are shown, with the letter indicated in a circle. Each dot represents a separate pole. The boxed number near a warren number indicates the number of poles located at warren entrances. The position of the observation hide is shown.

Samples were taken from every pole for five days, in late October and early November 1996 (October 29, 1996, November 5–8, 1996). This is late in the breeding season of rabbits at the Cattai site. There was no rainfall during this period, and observations during spotlighting and from a hide suggest that no other mammal species are likely to have contaminated the samples by also marking the poles. Therefore, the samples collected from these poles are attributable to rabbit scent marks.

The sample layer of foil was removed from the poles early in the morning of the sampling day. The foil was placed in an airtight headspace vial (10 ml), and the vial was closed with an airtight Teflon-faced rubber seal. The foil on the chinning pole was replaced, that is, the foil was left on the pole for 24 hr. Control

foil samples were also collected from each pole on each sampling occasion and treated in the same manner to compare with the experimental foil samples.

The foil samples were returned to the laboratory, and prepared in the same way as used previously for cotton swabs of the rabbit chin region (Hayes *et al*., 2001a). This dynamic headspace sampling trapped the volatile components of the secretion onto a sample of Tenax TA in a trap. The traps were analyzed on the PTV-GC to ascertain the volatile components. Analysis was conducted using a Hewlett Packard 5890 Series II gas chromatograph (GC) equipped with a flame ionization detector and fitted with a silica capillary column (SGE, model BP-1, 25 m long $\times$ 0.22 mm ID and 0.1-μm film thickness). Data were acquired under the following GC conditions inlet temperature: 200°C, carrier gas: nitrogen at 23 cm/sec, split ratio: 1:20, detector temperature: 260°C, temperature program: initial temperature 45°C, initial time 5 min, rate 8°C/min, final temperature 250°C, final time 10 min.

The resultant chromatograms were compared to those previously obtained, and peak numbers assigned as previously discussed (Hayes et al., 2001a). The chromatograms were compared specifically with swabs taken from animals belonging to the relevant social groups. The data (relative areas under each peak) were analyzed by Bray-Curtis similarity cluster analysis to determine if any groups were formed.

Marks on Rabbits. Swabs were taken from the top of the head of all animals trapped, in a manner similar to that used for collection from the chin region (Hayes et al., 2001a). The fur was not clipped from the top of the head. The cotton bud was swabbed 20 times over the area with a firm pressure. The swab was then placed into an airtight vial, stored in a freezer at -20°C, and analyzed by dynamic headspace gas chromatography (see above and Hayes et al., 2001a). The chromatogram produced was compared to the chin swab from the donor, as well as the swab from the chin of the dominant male from the same warren. The head region was chosen for collection, as an animal presumably cannot chin-mark its own head. Therefore, any mark that was collected from this area was assumed to have been placed there by another individual.

Social status of the wild animals was determined in several ways. For those social groups that lived near the observation hide, direct observations of displacement behavior and other agonistic interactions were possible. For rabbits from other parts of the site, indirect methods of assessment had to be employed. These included: body mass, encrustation of the chin region, the size and date of descent of the testes (since testes are withdrawn into the body outside of the breeding period), and knowledge of other rabbits living in the social group. Thus, a dominant male was the largest male trapped on the warren, with the largest testes, that descended early in the breeding season, and with a large amount of secretion encrusted in the chin region. An animal that was subordinate was taken as a small animal, living in a warren with larger males. The testes of this rabbit were small and descended late in the breeding season; the rabbit also had little or no encrustation on the chin.

Head and chin swabs were examined from 10 pairs of dominant males and subordinate individuals, each pair from a different social group. The chromatograms were examined to determine whether or not the head swabs of the subordinates resembled the chin swabs from the dominant animals more than chin swabs from themselves.

RESULTS

Environmental Marking. Only 23 of the 174 poles located on the site showed a chromatogram that differed significantly from control samples (ANOVA, $F = 350.3$, $P \ll 0.001$). The chromatograms obtained from the other poles are indistinguishable from both a sample of aluminum foil and a control sample. Of those that were marked, 20 had a mark on every collection, while the other three showed marks on four of the five collection days. Of the 870 poles that were available for marking across the experiment, only 112 ever showed a mark. This is significantly different to random when compared to a Poisson distribution ($\chi^2 = 86.4$, $P < 0.001$)

Although there were some minor variations within the control chromatograms, they all contained significantly fewer peaks (4.22 ± 0.23) than the samples considered to have been marked (17.6 ± 0.69). The chromatogram of a control sample is illustrated in Figure 2. However, the 23 poles that regularly showed a scent mark over the five collection days contained many peaks that did not occur in the control (Figure 3). The additional peaks had retention times mostly between 15 and 20 min. There were 17 compounds found at least once in the experimental marks, but never in the controls (Table 1). Of these, eight occur in all of the samples.

The swabs from the rabbits show the presence of siloxane compounds that are not present in the blank swabs. While these compounds are generally considered to be contaminants, exactly why these peaks do not appear in the blank samples is not clear. As they do not occur in the control or blank samples, they have tentatively been included in subsequent analyzes, although their exact relevance is not certain.

All the poles that showed a scent mark included 2-phenoxyethanol as one of the constituents. This compound has been shown to be associated with social status in the European rabbit, only occurring in the secretion of a dominant male and never in the secretion of subordinate animals (Hayes et al., 2001b). As all marks contain this compound, there is no evidence for marking by any individual other than the dominant male.

A K-means cluster analysis distinguishes the scent marks into two groups with a squared distance between the clusters of 13.998 (Table 2). The 10 most important peaks in assigning a sample to a cluster are listed (Table 3).

There was no correlation between the marks on poles and the chromatograms of chin swabs taken from animals on the same warrens. The signals from specific

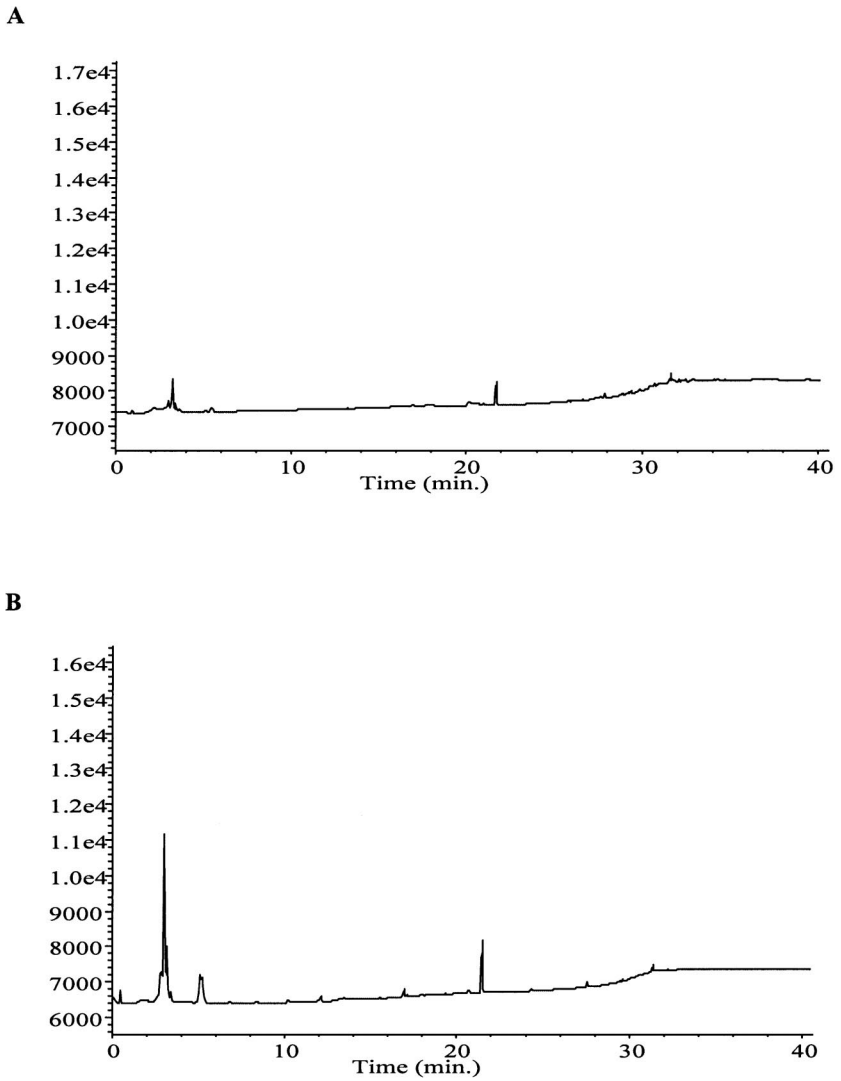


FIG. 2. Chromatogram of (A) blank sample of aluminum foil; and (B) control chinning pole (pole b4 control).

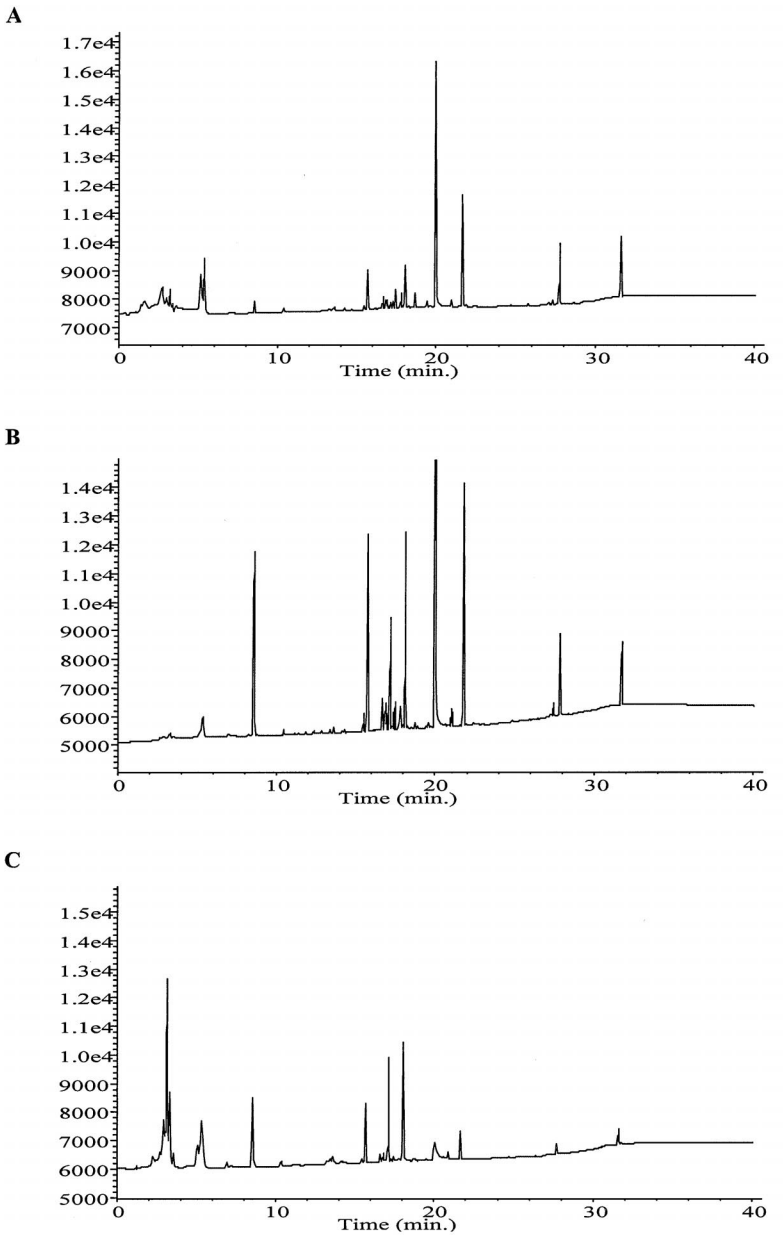


FIG. 3. Chromatograms of sample chinning-poles that contain a scent mark, showing the additional peaks. (A) pole c3; (B) pole f9, and (C) warren pole 66c.

TABLE 1. COMPOUNDS FOUND IN EXPERIMENTAL SAMPLES BUT NOT IN CONTROLS[a]

Compound number	Compound name
7	
14	1,3-dimethylbenzene
16*	siloxanes
38	α-pinene
20*	benzaldehyde
39*	siloxanes
41*	siloxanes
40	
22*	1,2,4-trimethylbenzene
23	
24	
25*	2-ethylhexanol
50	4-methyl-1-undecane
26	
29*	hydrocarbon
51	
45*	2-phenoxyethanol

[a] Starred compounds occur in all of the samples, compounds without a name could not be positively identified. The compound number refers to previous studies on the European rabbit (Hayes et al., 2001a).

TABLE 2. SAMPLE CLUSTERS[a]

Cluster 1		Cluster 2	
c3	i9	e12	h4
c6	i18	f12	h6
f9	k11	f13	h11
g13		g1	w66c
i1		g3	w76a
i3		g5	w78a
i8		h2	

[a] Samples that showed a scent mark could be subdivided into two clusters by a K-means cluster analysis, with a squared distance between the clusters of 13.998. The letter in a sample name refers to the chinning pole line, and the number refers to its position along that line. Sample names starting with a "w" are those poles that are located on a warren, referred to by warren number.

TABLE 3. TEN MAJOR COMPOUNDS USED TO ASSIGN POLES TO ONE OF TWO CLUSTERS IDENTIFIED

Compound number	Compound name
12	dimethylbenzene
15	xylene
16	siloxanes
17	siloxanes
18	2,4,6-trimethyloctane
20	benzaldehyde
39	siloxanes
22	1,2,4-trimethylbenzene
25	2-ethylhexanol
28	1-methyl, 4-isopropylbenzene

poles do not look like signals from individual rabbits. A cluster analysis of chin swabs and pole marks separates these two groups of chromatograms as being different from each other.

The location of marked poles is not random (Figure 4) ($G_H = 16.212$, $P < 0.005$) (Sokal and Rohlf, 1995). There are more marked poles close to warrens

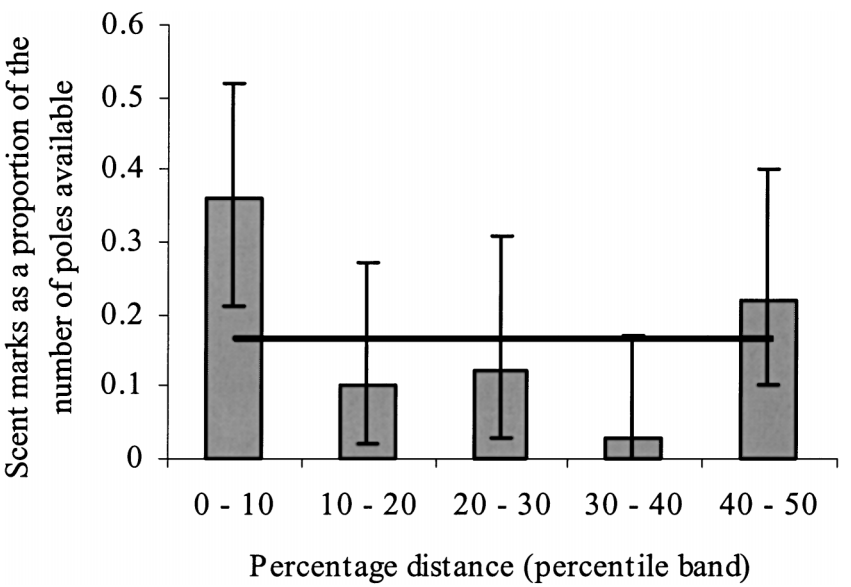


FIG. 4. Percentage distance of marks between two warrens as a proportion of the number of poles available. The dark line is a plot of the mean value (error bars are 95% confidence limits of the binomial distribution).

than there are in other parts of the territory, and the number of marks located midway between adjacent warrens also is higher than the mean level of marking throughout the territory. This location corresponds to the boundary between social groups as ascertained from the scratch marks observed on the site. The numbers of marks found on warrens and midway between groups are statistically homogeneous ($G_H = 1.698$). The rabbits are actively scent marking at the warrens and at the edge of their group territory more than they are at other locations within the territory.

Marks on Rabbits. There is a large difference between the head and chin swabs from a dominant individual (Figure 5). The number of peaks detected from a head swab (9 ± 1.0) is less than that detected from a chin swab (22 ± 2.1). Both the animals shown have very little signal on their head, but the signal collected at the same time from the chin has many more peaks in the chromatogram. This is also true of the other eight dominant male head swabs examined. 2-Phenoxy ethanol, the dominant signaling compound, was never detected from the head swab of a dominant animal.

There is also a difference between the head and chin swabs of subordinates and females. In this case, however, there are many more peaks identified from the head than from the chin (Figure 6).

In a comparison among the pairs of 10 subordinate and dominant individuals, each pair from the same warren showed that the signal from the head of the subordinate was similar to the chin signal from the dominant male (Figure 7). It was not the same as the signal from the donor's own chin swab. MDS analysis (Clarke and Warwick, 1994) groups together the swabs from the dominant's chin and the subordinate's head more closely than the swabs from the subordinate's chin and head (ANOSIM: Global $R = 0.478$, $P < 0.001$) (Figure 8).

DISCUSSION

Environmental Marking. While it is clear that a specific set of poles has been marked, it is not possible to recognize the signal of the dominant male on the chinning poles located on his warren. However, the data support the hypothesis that there is structuring of the marking behavior on the warren. There are more marks located on poles that are placed next to active warren entrances or distributed in this immediate vicinity. There are also more scent marks found at the proposed boundary between adjacent social groups. This produces an olfactory bowl, similar to that seen in the wolf (*Canis lupus*) (Peters and Mech, 1975).

There was no evidence of scent marks on poles from any individuals other than the dominant male (as determined by presence of 2-phenoxy ethanol); the scent mark of the dominant females present was not detected on the poles at warren entrances, as was hypothesized. This may be due to the nature of the female scent mark. If the mark of a female were overmarked by the dominant male in her social

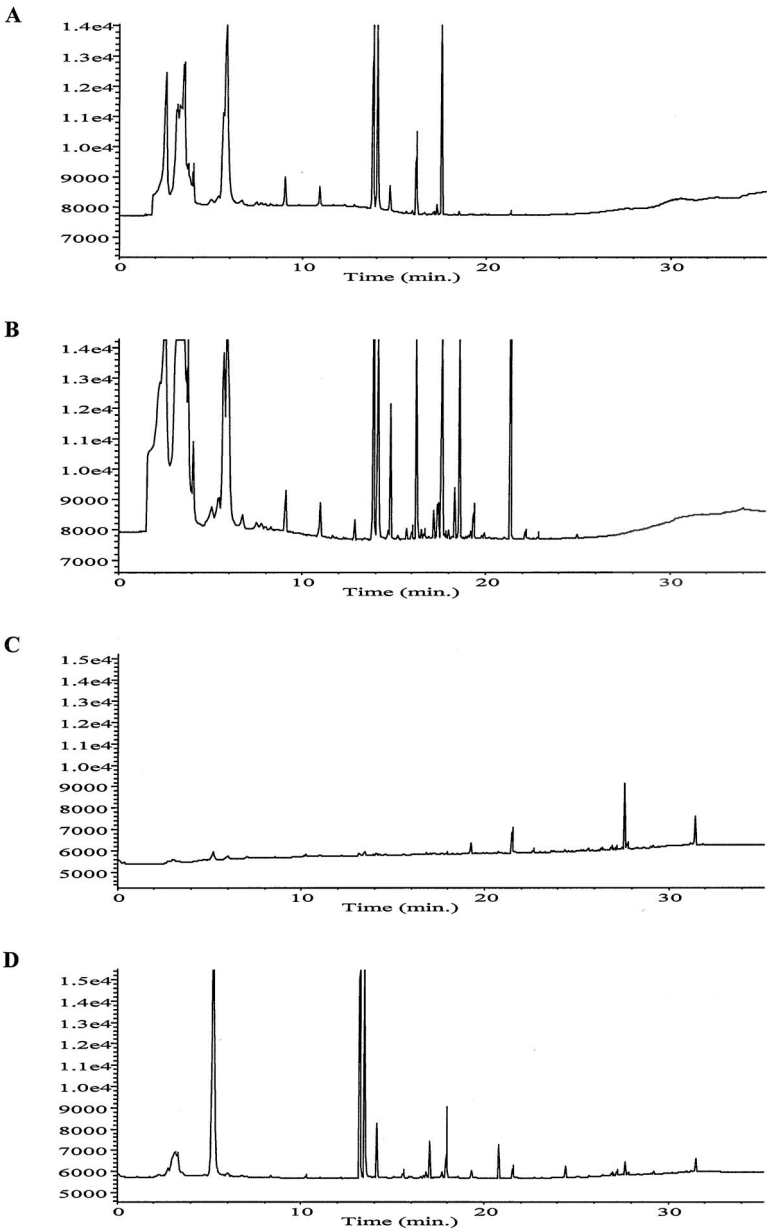


FIG. 5. A comparison of the head and chin swabs of dominant male rabbits showing the reduced presence of volatiles on the head suggesting a lack of a scent mark. Chromatograms are: (A) O + Bk/O 12/12/96 head; (B) O + Bk/O 12/12/96 chin; (C) R + W/G 7/9/95 head; and (D) R + W/G 7/9/95 chin.

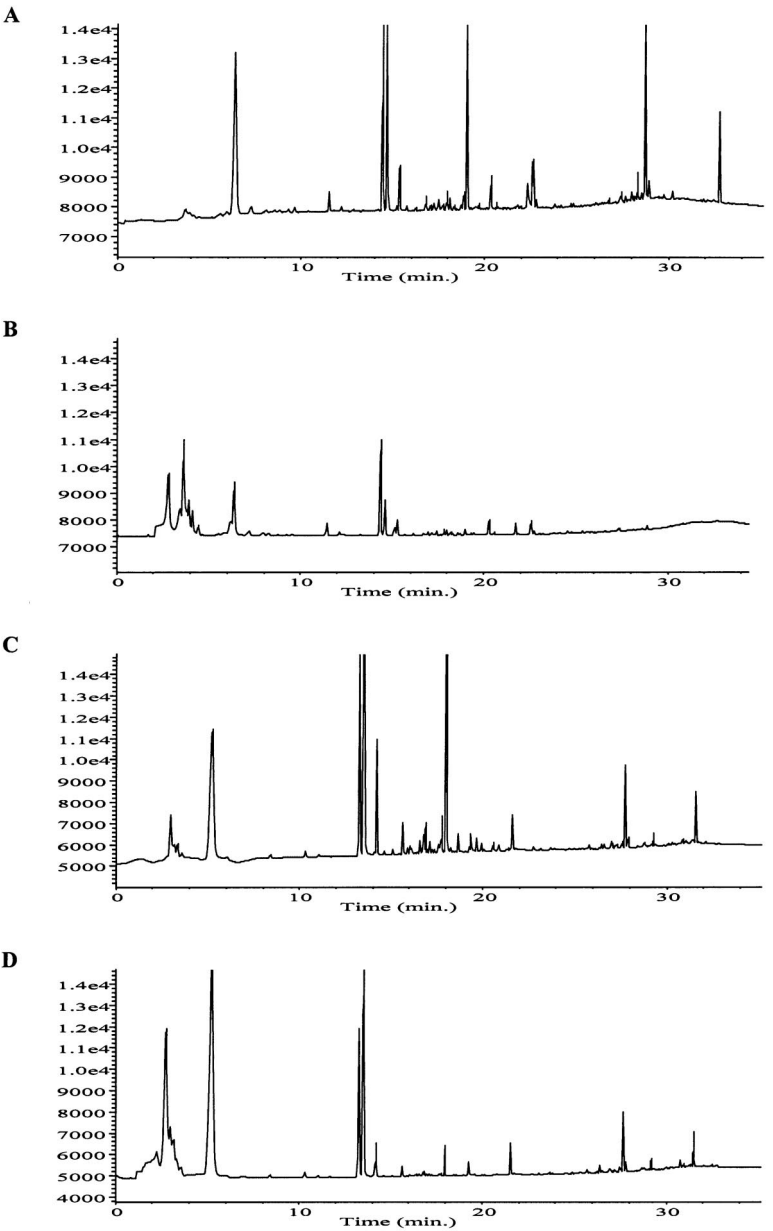


FIG. 6. A comparison of swabs from the head (A and C) and chin (B and D) of some subordinate rabbits. Note that the signal from the head contains more peaks than that from a swab of the chin region. Chromatograms are: (A) Pi + Bk/Pu 30/10.95 head; (B) Pi + Bk/Pu 30/10/95 chin; (C) Pu + W/G 6/10/95 head; and (D) Pu + W/G 6/10/95 chin.

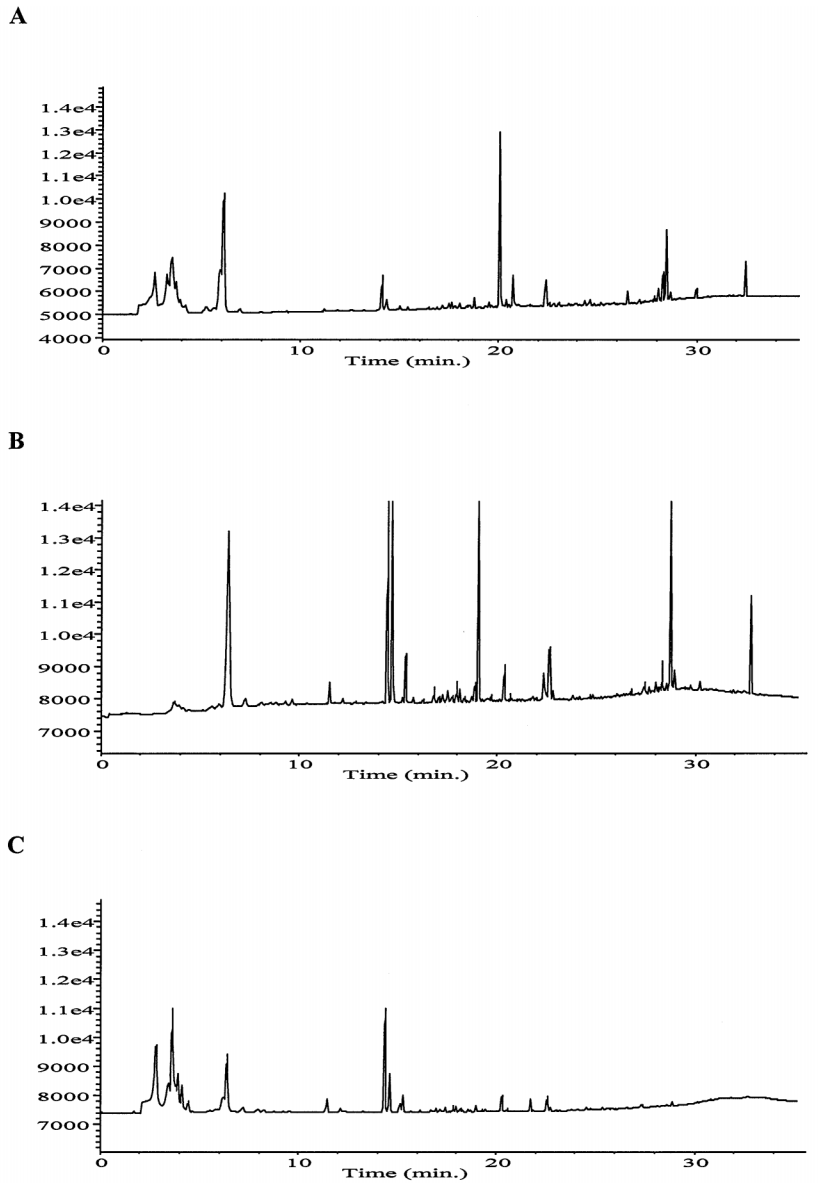


FIG. 7. A comparison between the head and chin swabs of dominant and subordinate rabbits from the same warren. The top chromatogram (A) is from the chin swab of the dominant individual (R + Bk/O 15/11/95). The lower chromatograms are from the head (B) and chin (C) from the subordinate individual (Pi + Bk/Pu 30/10.95).

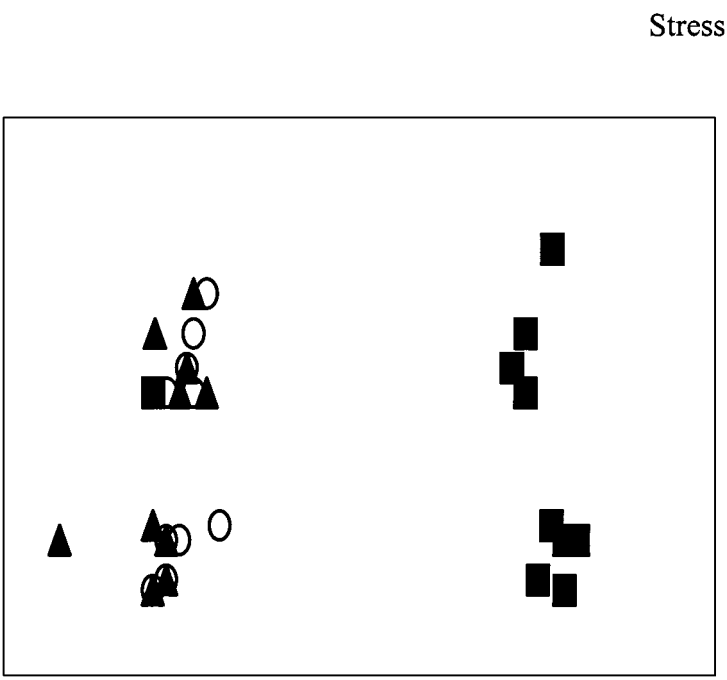


FIG. 8. The two-dimensional MDS ordination of the 30 samples, based on square root transformed abundances and a Bray-Curtis similarity matrix. Distinct clusters emerge showing grouping of the swabs from the dominant's chin and the subordinate's head against swabs from the subordinate's chin. ○ = samples from dominant chin swabs; ■ = samples from subordinate chin swabs; ▲ = samples from subordinate head swabs.

group, then her mark would almost certainly not be detected because there is much more secretion from the gland of a male than from a female and it appears to be more concentrated (Mykytowycz, 1965; Mykytowycz and Dudzinski, 1966). The marking behavior of the male may, thus, be masking the female marking. This does not mean that the female's mark is undetectable to a rabbit, merely that the techniques employed in the present study do not detect them.

It is reported that females mark at the entrances to burrows that contain their unemerged kittens (less than 28 days old) (Mykytowycz, 1968; Gibb et al., 1978). We know from trapping at this site that there were kittens living below ground in the warrens in question during the period of study. If the reported female marking occurred at Cattai, then it must be that we are unable to detect the female's mark. Since the females are suggested to mark the dirt immediately adjacent to the warren entrance, it is probable that the pole is not marked. The other marking behaviors of the female at the warren entrance, i.e., urination and defecation (Mykytowycz, 1968) may play a more significant role.

The scent marks detected on the poles across the site can be divided into two groups, and these clusterings are distinguishable statistically. The patterns seen are not due to differences in the presence or absence of compounds, but are related to changes in the relative proportions of the constituents. The poles belonging to each group are also geographically clustered. The two clusters are not just the marks from two individuals. These groupings cover several social groups, with dominant males trapped from four or five separate warrens within the area covered by each cluster. Previous work at this site has shown that the deme size is 100 m (Richardson, unpublished results), approximately the difference between these two groups. It is possible that these clusters represent a deme, and within this group the animals are more closely related to one another than they are to animals from outside the area. If the difference is genetically determined, then this pattern of distribution would not be incompatible with movement of the males at these population densities (Richardson et al., 2001). This relatedness may show in the similarity of the chin secretion, as detected on the poles. However, any conclusions about these data should be drawn carefully, and further investigation is required before anything can be ascertained with confidence.

The placement of the chin marks in the rabbit territory did not agree with expectation. Marks were preferentially located at the boundary, and the placement was inversely related to distance from the center of the territory. This does not conform to the proposal set forth by Mykytowycz (1965), in which he suggested that rabbits would mark in a random manner, such that the environment would be saturated with scent. There is also nonrandom marking of a restricted set of poles. Once a pole has been marked, that pole continues to be marked throughout the period of the experiment. Marking sites were overmarked.

One explanation for the observed distribution of the chin marks is that the rabbit's marking behavior is highest on the warren, then as distance increases away from the core territory, the frequency of marking decreases. That is, the frequency of marks is inversely proportional to distance from the warren. To this must be added a boundary effect at the edge of the territory controlled by a social group. Although the amount of marking by members from each group individually is low, the numbers of marks on both boundaries add together, and the observed frequency is, therefore, artificially high. Another explanation could be that rabbits make a choice about where to mark. Individuals mark preferentially on the warren and at the edge of the area controlled by the social group. Marking that occurs between these two extreme locations is at a much lower frequency than that seen at either one. This pattern is seen in badgers (Roper et al., 1993).

These two explanations rely on different perceptions of space by the rabbits. The first sees the core territory as being most important. As you move further away from this area, the need to alert others of the ownership of the site is reduced. The further from the core zone one is, the less important this communication becomes. The second hypothesis has the rabbits perceiving the whole of the controlled

territory as being important; however, it is principally at the edge of this territory that warnings to others to stay away need to be placed. Any rabbits that occur within the social group's boundaries must either belong to the social group or have had to cross a boundary to enter and must consequently have encountered the message. Invaders will also be progressively more likely to encounter a message the further they penetrate into the territory.

Data from the published literature suggests that the second hypothesis is more likely to be accurate. Dominant males are known to chin mark at the entrances to warrens (Mykytowycz, 1968), as are females (Gibb et al., 1978). Wild rabbits are known to concentrate activities on the edge of the territory controlled by the social group, including paw-scraping, defecation, and chin marking (Mykytowycz, 1968; Bell, 1980; Cowan and Bell, 1986). Thus, the rabbits "know" where the centers and boundaries of their territories are and actively defend them both.

A further explanation may be that animals do not choose where they will place their scent marks, but merely mark wherever they are. Thus, the observed distribution of marks is a product of where animals are spending the majority of their time, rather than the result of specific decisions to mark the warren and boundaries of their territory. This explanation would seem unlikely, as dominant male rabbits are observed to actively tour the extent of their territory (Mykytowycz, 1968) and, thus, seem to be making a decision to mark in these locations.

Several of the pole lines were extended into communal feeding areas (line A, B & J), where no social group is in control, and no marks were detected on these poles. Thus, in the communal areas used by all animals from adjoining territories, no one animal is in control, and no individual leaves a scent mark. This also raises the interesting point that rabbits are believed, as reported above, to mark any novel object placed within their sphere of influence (Mykytowycz, 1967, 1968, 1972; Johnson, 1973). However, as the present study shows, the majority of poles placed within the group territory were not marked. The explanation for this may be that previous studies have all considered animals kept in cages or enclosures. The restricted space available in these systems may have affected the behavior.

The marks that occur at the entrances to warrens are likely to serve a different function. Instead of being a message to warn members of another social group that they are entering an area already under the control of a group, they may serve as an intragroup message.

It is not possible for the experimenter to distinguish the scent marks collected on the poles from one another, except in a fairly superficial way into two broad groups. This means that if the marks found on a warren are carrying a different message from those found at the boundaries, it is not detectable by current analytical methods. This lack of distinctiveness may be due to the method of collection. Samples were collected onto aluminum foil, a substance that has vastly different properties from the usual materials that are used to carry a rabbit's scent mark. The highly reflective surface of the foil, and the much higher heat conductivity may

well mean that some components of the secretion will not adhere to the surface or that some of the constituents may be lost more quickly than would occur from a more typical surface (Reigner and Goodwin, 1977). However, animals on this site come above ground no earlier than about 1 hr before sunset. Since the foil was collected from all poles before 9:00 AM, the samples were not exposed to sunshine for a very long period, and there seems to be little time for this evaporation to occur. In addition, for at least some of the more common constituents of rabbit chin gland secretion, aluminum foil is suitable and it retains the compounds as well as a biological substrate (Hayes, unpublished data).

Another explanation may be that the marks on poles might actually not be different from each other. Rabbits may produce two types of signals from the chin gland. One may be used to mark the territory and establish that this area belongs to a dominant individual. This is the signal found on the chinning poles. The other could be used to mark individuals as belonging to that group. This signal would need to be individually identifiable; a generic dominant signal would not suffice. This second type of signal would be that collected from a chin swab and also from the head of other animals in the social group (see below). There is no histological evidence of two separate glands in the chin region that would allow this division of the signal; however, there are three types of cells (Lyne et al., 1964), and, thus, it is possible that there is more than one type of signal produced by the dominant male.

The marks on poles and the head and chin swabs were collected from the same habitat at the same time, and yet the swabs from an animal's skin show the full complexity of peaks, while all the poles show a depauperate signal. Either this difference detected among the samples is trivial, an artifact of the collection technique, or it reveals something more important: that there are two types of mark produced by the rabbit chin gland.

Marks on Rabbits. Dominant male rabbits are known to place their scent marks on other individuals in their social group (Bell, personal communication, Mykytowycz, 1968). It is possible to identify the secretion of the dominant male from the swab taken from the head of a subordinate individual. It is also possible to ascertain the social status of an individual without reference to any behavioral observation, but instead from the pattern of secretion found on its head and chin. If an animal is dominant, then his chin swab will contain more peaks than the swab from his head. No other animal is dominant to him, and, thus, there is none that places a scent mark upon his head. If, on the other hand, the animal is a subordinate individual, then the swabs collected will show the opposite pattern. The head swab (the mark of the dominant individual) will be more complex than the swab from under the chin.

It is also possible to determine the composition of the secretion from the dominant individual in a social group without having to trap this male, simply from the swab of the head of a subordinate animal. Since dominant animals are notoriously

trap-shy (Mykytowycz, 1964; Sunnocks, 1998), this gives the opportunity to collect this information from any social group, whether or not the dominant individual is trapped. A mark that is collected from the head of a rabbit is the scent mark of the dominant male of that social group, unless the donor animal is the dominant male, and then no mark is detectable on the head.

There is a structuring of the location of scent marks through the territory controlled by a rabbit social group. There are significantly more marks placed near the warren and at the borders with other social groups, than there are throughout the rest of the territory. This suggests that the rabbits are using a semiochemical message to defend the core of the territory, and the boundaries.

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CUTICULAR HYDROCARBONS AND NOVEL ALKENEDIOL DIACETATES FROM WHEAT STEM SAWFLY (*Cephus cinctus*): NATURAL OXIDATION TO PHEROMONE COMPONENTS[1]

ROBERT J. BARTELT,[2,]* ALLARD A. COSSÉ,[2] RICHARD J. PETROSKI,[2] and DAVID K. WEAVER[3]

[2]*USDA Agricultural Research Service*
National Center for Agricultural Utilization Research
Crop Bioprotection Research Unit
1815 N. University Street
Peoria, Illinois 61604

[3]*Montana State University*
Department of Entomology
P.O. Box 173020
Bozeman, Montana 59717-3020

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Abstract—The cuticular lipids of the wheat stem sawfly *Cephus cinctus* (Hymenoptera: Cephidae) were investigated as part of a chemical ecology project with this species. The major cuticular lipids were *n*-alkenes and *n*-alkanes. Alkenes were the most abundant and exhibited dramatic sexual dimorphism. (*Z*)-9-Tricosene accounted for about half of the total hydrocarbon in males but was nearly absent from females. The dominant alkenes in females were (*Z*)-9-pentacosene and (*Z*)-9-heptacosene. The alkane profiles were similar in both sexes, with *n*-tricosane being the most abundant, followed by *n*-pentacosane and *n*-heptacosane. In both sexes, there were minor amounts of alkanes and alkenes with other chain lengths and *n*-alkadienes of 29 and 31 carbons. In males, about one tenth of the surface lipids consisted of (*Z*)-9-alkene-1, ω-diol diacetates with 22-, 24-, and 26-carbon chains. The same compounds were also detected from females but in much smaller amounts. The structures of these novel diacetates were proven by synthesis. By analogy to methyl oleate, a well-studied food lipid, the alkenes and diacetates were expected to undergo slow oxidation in air to release specific aldehydes and other volatile products, and these were generally detected in volatiles collected from living sawflies.

* To whom correspondence should be addressed. E-mail: BartelRJ@NCAUR.USDA.gov

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Atmospheric oxidation of the diacetates was also demonstrated in the absence of sawflies. One product from the diacetates, 9-acetyloxynonanal, was shown in other research to be particularly active electrophysiologically and was also attractive in the field. Aldehydes from the alkenes also showed strong electrophysiological activity. The concept of volatile pheromones originating from heavy, unsaturated cuticular lipids is discussed.

Key Words—*Cephus cinctus*, wheat stem sawfly, pheromone, cuticular lipid, hydrocarbon, alkene, alkane, (*Z*)-9-docosene-1,22-diol diacetate, (*Z*)-9-tetracosene-1,24-diol diacetate, (*Z*)-9-hexacosene-1,26-diol diacetate, aldehyde, oxidation.

INTRODUCTION

The wheat stem sawfly *Cephus cinctus* Norton (Hymenoptera: Cephidae) remains an important pest of wheat in the northern Great Plains, and the chemical ecology of this species is now being investigated with the goal of finding new management tools. Aspects of this research have included the chemical identification, electrophysiological activity, and attractiveness of volatile compounds from the sawfly (Cossé et al., 2002). Compounds from sawfly volatiles collections that elicited strong electrophysiological responses included 9-acetyloxynonanal, nonanal, tetradecanal, phenylacetic acid, and other aldehydes and carboxylic acids. 9-Acetyloxynonanal was attractive to both sexes in preliminary field tests (Cossé et al., 2002).

In another sawfly species, *Pikonema alaskensis* (Rohwer) (Hymenoptera: Tenthredinidae), the volatile long-range pheromone also includes aldehyde components, and these originate in an unusual way, through the oxidation of essentially nonvolatile cuticular lipids (Bartelt and Jones, 1983). The cuticular lipids of *C. cinctus* were, therefore, studied to explore whether such a mechanism might operate in this species. The results suggest it does. This report includes a survey of the cuticular lipids of *C. cinctus*, the synthesis of three novel constituents to prove structure, and a discussion of the production of volatile pheromone components from unsaturated lipids by oxidation.

METHODS AND MATERIALS

Extracts and Collections of Volatile Compounds. Hexane extracts of whole, individual sawflies were prepared in Montana by one of us (D.K.W.) and sent to Peoria for analysis. The insects were field collected as prepupae and handled as described by Cossé et al. (2002). Individual adult sawflies were extracted for either 20 min or 24 hr in ca. 0.5 ml hexane, and the sex and age of each insect was recorded. Because mating occurs soon after emergence in the field, the chosen age

categories emphasized the younger insects: 1, 2, 3, 4, and 5 hr after emergence, 1–2 days old, and 5–6 days old. Some sawflies were also extracted before emergence; these were removed from the wheat stem stubs after they had eclosed from their pupae but before they had chewed through the plug in the stem. A total of 23 extracts of individuals were analyzed by GC-MS and compared. Later, additional hexane extracts were prepared in Peoria from insects received from Montana. These included 1 sec dips and 24 hr soaks of groups of 10–25 male or female sawflies.

The collection of volatiles from living sawflies was described by Cossé et al. (2002). The GC-MS data files for these previous volatiles collections were studied further for additional information.

Instrumentation. The details of the GC-MS system were given by Cossé et al. (2002). Briefly, the HP 5973 Mass Selective Detector was interfaced to an HP 6890 GC. Cuticular lipid samples were analyzed on a thin-film DB-1 GC column (15 m length, 0.25 mm ID, 0.1 μm film thickness; J & W Scientific, Folsom, California). The oven program started at 50°C for 1 min, increased at 10°C/min to 300°C, and remained at 300°C for 5 min. The inlet and transfer line were held at 300°C and 250°C, respectively. The usual MS scanning range was 40–550 amu (EI, 70 eV). The Wiley mass spectral library with 275,821 spectra (Wiley, 1995) was available on the MS data system. Quantitation of cuticular components was done by the external standard method on an HP 5890 Series II gas chromatograph equipped with flame ionization detector. High-performance liquid chromatography (HPLC) was used to separate geometrical isomers of alkenes and of one alkenediol diacetate. The system consisted of a Waters M6000 pump, Waters R401 differential refractometer detector, and $AgNO_3$-treated column. The $AgNO_3$ column was prepared from a 25-cm $\times$ 4.6-mm-ID silica column (Alltech, Adsorbosphere, 5 μm) by the method of Heath and Sonnet (1980). The elution solvent was 0.25% 1-hexene in hexane for alkene hydrocarbons and benzene for the alkenediol diacetate. HPLC on a silica column (Alltech, Econosphere Silica 5μ) was also used to separate the sawfly-derived alkenediol diacetates from the other cuticular lipids (10% ether in hexane). Infrared spectra of purified synthetic alkenediol diacetates were obtained as thin films on KBr plates using a Nicolet Impact 410 FT-IR spectrometer. The proton NMR spectrum was obtained at 400 MHz for a synthetic alkenediol diacetate on a Bruker Avance 400 instrument. The solvent was deuterobenzene, and the shifts were measured relative to TMS.

Dimethyl Disulfide Derivatives. Dimethyl disulfide (DMDS) adducts were prepared from the sawfly-derived olefinic lipids and the synthetic alkenediol diacetates so that locations of double bonds could be determined (Carlson et al., 1989). Briefly, the samples were evaporated to dryness under a stream of nitrogen, and DMDS and 5% iodine in ether were added (equal volumes, about 25 μl each). The samples were heated at 45°C for 1–2 hr, then diluted with hexane and worked up with aqueous sodium thiosulfate to destroy the iodine catalyst. The hexane layer, which contained the products, was dried over sodium sulfate, evaporated under

nitrogen, resuspended in about 10 μl of hexane, and analyzed by GC-MS. The MS scanning range for these was 40–650 amu.

Synthesis of Alkene-1, ω-Diol Diacetates. Three alkenediol diacetates (**8**, **9**, and **10**) were synthesized by one of us (R.J.P.), using standard methods, to confirm chemical identifications (Figure 1). The Wittig reaction was the key synthetic step, and most of the reactions in Figure 1 involved the creation of the required Wittig salts and aldehydes from commercially available starting materials and the addition or removal of protecting groups. The Wittig salts (**1–4**) were prepared from 9- or 11-carbon bromohydrins (Aldrich, Milwaukee, Wisconsin) by refluxing with triphenylphosphine in acetonitrile, followed by solvent removal and trituration with dry ether to remove impurities. For two of these (**1** and **2**), the hydroxyl group

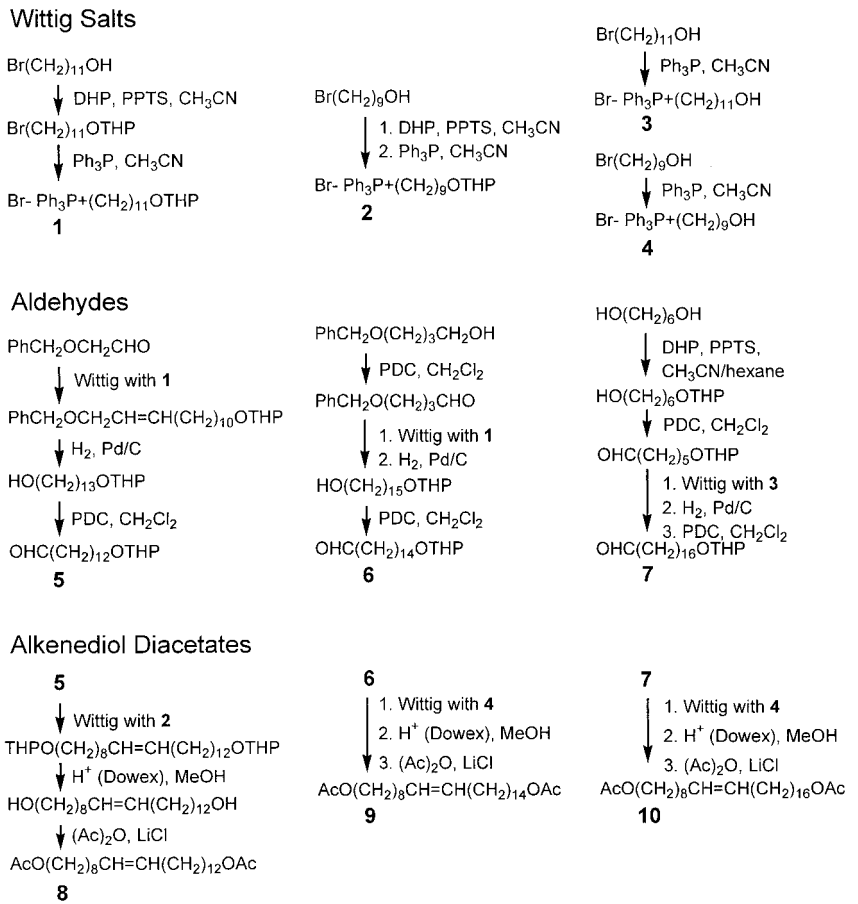


FIG. 1. Synthesis of the 9-alkene-1,ω-diol diacetates by Wittig routes (see text for abbreviations).

was protected as a tetrahydropyranyl (THP) ether before salt formation by reaction with 2,3-dihydropyran (DHP) in acetonitrile, using pyridinium *p*-toluene sulfonate (PPTS) as catalyst (Miyashita et al., 1977).

The carbon chains of the aldehydes (**5**–**7**) were all built up from smaller fragments with Wittig reactions (Sonnet, 1974). For **5** and **6**, two- and four-carbon benzyl-ether aldehydes were condensed with the 11-carbon THP Wittig salt. The four-carbon aldehyde was not commercially available but was made from its alcohol by oxidation with pyridinium dichromate (PDC) (Corey and Schmidt, 1979). Hydrogenation of these Wittig products over 10% palladium on carbon both removed the benzyl protecting group (but not the THP group) and reduced the double bond (Greene and Wuts, 1991). The free hydroxyls were subsequently oxidized to aldehydes with PDC. To make aldehyde **7**, the six-carbon diol was first converted to a mono-THP ether in a biphasic mixture of acetonitrile and hexane; the reactants were primarily in the more polar (acetonitrile) phase, but the mono ether migrated to the hexane phase, reducing the chance of further derivatization to the diether. The free hydroxyl was then oxidized to an aldehyde, and the aldehyde was condensed with hydroxy Wittig salt **3**. The double bond of the resulting 17-carbon product was hydrogenated over palladium on carbon, and the final aldehyde was formed by PDC oxidation of the alcohol.

The final Wittig condensations with the prepared aldehydes involved either the protected (THP) or hydroxy salts; both gave the expected products, although the yields in either case were only fair. The THP groups were removed in MeOH under acid catalysis (provided by Dowex 50W-X4 ion-exchange resin, which was later easily removed by filtration) (Beier and Mundy, 1979). Finally, the free alkenediols were acetylated with acetic anhydride, using lithium chloride as the Lewis acid catalyst (Sabitha et al., 1999).

The synthetic requirement was only to obtain enough material for chromatographic and spectral comparison with the natural material. Reactions were not optimized for yield, and in most cases, intermediates were used in subsequent reactions without purification. Overall yields were less than 1% from the starting materials, but the desired final products were distinct and readily recognizable in the reaction mixtures.

Reactions were monitored by mass spectrometry. Mass spectral and GC retention data for the syntheses in Figure 1 are summarized in Table 1. Mass spectra were not obtained for the Wittig salts **1**–**4**. The THP ether and diol intermediates for **9** and **10** were not analyzed by GC-MS but were converted directly to the diacetates before analysis. Synthetic **8**–**10** consisted of both *E* and *Z* isomers, which were separable by GC. Synthetic **10** was investigated further so that the proper configuration could be assigned to each GC peak. HPLC on the $AgNO_3$/silica column was used to separate the geometrical isomers, and infrared spectra were then obtained for these. A proton NMR spectrum was also acquired for the more abundant isomer.

TABLE 1. GC RETENTION TIMES AND MASS SPECTRAL DATA FOR INTERMEDIATES AND FINAL PRODUCTS IN SYNTHESIS OF 1,ω-ALKENEDIOL DIACETATES (SEE FIGURE 1)

Compound and (GC retention in min.)	Mass spectral fragments [m/z] and (relative abundance)
Intermediate for compound **1**	
$Br(CH_2)_{11}OTHP$ (15.92)	85 (100), 101 (8), 333 (2, M), 335 (2, M)
Intermediate for compound **2**	
$Br(CH_2)_9OTHP$ (13.98)	85 (100), 101 (10), 305 (3, M), 307 (3, M)
Intermediates for compound **5**	
$PhCH_2OCH_2CH{=}CH(CH_2)_{10}OTHP$ (21.57, 21.73)[a]	85 (100), 91 (77), 101 (13), 107 (9), 303 (4, M-85), 387 (0.1), 388 (0.1, M)
$HO(CH_2)_{13}OTHP$ (16.92)	41 (16), 43 (11), 55 (24), 56 (16), 57 (13), 67 (9), 69 (18), 81 (4), 83 (13), 84 (10), 85 (100), 95 (3), 97 (9), 101 (31), 111 (4), 125 (1), 215 (0.7, M-85), 299 (0.5), 300 (0.1, M)
$OHC(CH_2)_{12}OTHP$ (**5**) (16.30)	41 (17), 43 (12), 55 (21), 56 (18), 57 (14), 67 (10), 69 (9), 81 (7), 83 (8), 84 (12), 85 (100), 95 (10), 97 (6), 101 (22), 109 (6), 123 (2), 213 (0.7, M-85), 297 (0.4, M-1)
Intermediates for compound **6**	
$PhCH_2O(CH_2)_3CHO$ (7.75)	91 (100), 107 (33), 178 (0.6, M)
$PhCH_{2O}(CH_2)_3CH{=}CH(CH_2)_{10}OTHP$ (22.83, 23.00)[a]	85 (100), 91 (62), 101 (5), 107 (7), 331 (6, M-85), 415 (0.1), 416 (0.4, M)
$HO(CH_2)_{15}OTHP$ (18.64)	85 (100), 101 (34), 243 (0.9, M-85), 327 (0.4), 328 (0.1, M)
$OHC(CH_2)_{14}OTHP$ (**6**) (18.10)	85 (100), 101 (27), 241 (1, M-85), 325 (0.4), 326 (0.2, M)
Intermediates for compound **7**	
$HO(CH_2)_6OTHP$ (9.57)	85 (100), 101 (32), 117 (3, M-85), 201 (0.3), 202 (0.1, M)
$OHC(CH_2)_5OTHP$ (8.60)	85 (100), 101 (25), 115 (1, M-85), 199 (0.4), 200 (0.1, M)
$HO(CH_2)_{10}CH{=}CH(CH_2)_5OTHP$ (19.94, 20.02)[a]	85 (100), 101 (2), 252 (1, M-84-18), 270 (0.8, M-84)
$HO(CH_2)_{17}OTHP$ (20.22)	85 (100), 101 (36), 271 (1, M-85), 355 (0.3), 356 (0.1, M)
$OHC(CH_2)_{16}OTHP$ (**7**) (19.73)	85 (100), 101 (27), 269 (1, M-85)
Intermediates for compound **8**	
$THPO(CH_2)_8CH{=}CH(CH_2)_{12}OTHP$ (26.61, 26.73)[a]	85 (100), 101 (11), 340 (1, M-84-84), 423 (1, M-85)
$HO(CH_2)_8CH{=}CH(CH_2)_{12}OH$ (20.42, 20.50)[a]	41 (51), 55 (100), 57 (25), 67 (69), 69 (60), 81 (71), 82 (94), 95 (63), 96 (69), 109 (32), 123 (16), 135 (10), 149 (4), 304 (1, M-18-18), 322 (3, M-18), 340 (1, M)

TABLE 1. CONTINUED

Compound and (GC retention in min.)	Mass spectral fragments [*m/z*] and (relative abundance)
$AcO(CH_2)_8CH{=}CH(CH_2)_{12}OAc$ (**8**) (22.08, 22.15)[a]	41 (34), 43 (100), 55 (81), 57 (15), 61 (21), 67 (71), 69 (52), 81 (75), 82 (74), 95 (67), 96 (66), 109 (37), 110 (30), 121 (25), 123 (20), 135 (19), 137 (11), 149 (8), 151 (5), 152 (5), 304 (4, M-60-60), 321 (3, M-60-43), 364 (3, M-60) 424 (4, M)
Compound **9**	
$AcO(CH_2)_8CH{=}CH(CH_2)_{14}OAc$ (23.43, 23.51)[a]	41 (34), 43 (100), 55 (85), 57 (22), 61 (24), 67 (65), 69 (59), 81 (74), 82 (70), 95 (64), 96 (63), 109 (36), 110 (29), 121 (22), 123 (21), 135 (17), 137 (12), 149 (7), 151 (5), 152 (6), 332 (4, M-60-60), 349 (3, M-60-43), 392 (4, M-60) 452 (5, M)
Compound **10**	
$AcO(CH_2)_8CH{=}CH(CH_2)_{16}OAc$ (24.75, 24.81)[a]	41 (33), 43 (100), 55 (85), 57 (22), 61 (25), 67 (65), 69 (61), 81 (74), 82 (72), 95 (67), 96 (64), 109 (36), 110 (30), 121 (20), 123 (22), 135 (16), 137 (11), 149 (7), 151 (5), 152 (5), 360 (4, M-60-60), 377 (2, M-60-43), 420 (4, M-60) 480 (6, M)

[a] *Z* and *E* isomers with nearly identical mass spectra. The spectral data correspond to the earlier, larger GC peak (*Z* isomer) in each case.

Other Standards. Samples of 8-acetyloxyoctanal and 10-acetyloxydecanal were prepared for comparison of GC retention times and mass spectra with components in sawfly volatile collections. The commercially obtained 8- and 10-carbon diols were converted to mono-THP ethers as described above, oxidized with PDC to mono-THP aldehydes, then deprotected and acetylated. The 13-, 15-, and 17-carbon aldehyde acetates were obtained by ozonolysis (see Cossé et al., 2002) of alkenediol diacetate samples. The saturated aldehydes of 6–14 and 16 carbons were either commercially available (6–14 carbons, Aldrich) or were prepared by ozonolysis of appropriate alkene hydrocarbons. Octanoic, nonanoic, decanoic, and phenylacetic acids were obtained commercially (Aldrich). Synthetic *E* and *Z* alkenes and various other alcohols, hydrocarbons, and 2*E*-alkenals were on hand from other research.

Laboratory Oxidation of Alkenediol Diacetates. Preliminary study of light-induced, abiotic oxidation of alkenediol diacetates was performed using a Suntest CPS solar simulator (Atlas, Gainesville, Florida), with the system set up as described by McGuire et al. (2000). Purified (HPLC) samples containing about 500 ng of synthetic **10** or natural male alkenediol diacetates were evaporated to dryness on the inside of quartz cuvettes. Cuvettes were capped and sealed with Teflon tape and placed in the solar simulator. The outside of the treated cuvette was in direct contact with the cooled (15°C) instrument surface. Samples were exposed for 2 hr

to light irradiation similar in intensity and wavelength composition (300–1100 nm) to sunlight. As a control, a sample of synthetic **10** was set up identically but without the light. All samples were resuspended in hexane after 2 hr and then analyzed by GC-MS.

RESULTS AND DISCUSSION

Cuticular Hydrocarbons. The cuticular hydrocarbons were the major compounds in the sawfly hexane extracts (Figure 2, Table 2). Both sexes had large amounts of *n*-alkanes and *n*-alkenes, primarily with odd numbers of carbons. The *n*-alkane profiles were similar between sexes, with tricosane being most abundant and with smaller amounts of 25-, 27-, 29-, and 31-carbon alkanes. There were traces of the intervening even-chain compounds and also the 21- and 33-carbon alkanes. No branched alkanes were detected.

The *n*-alkenes were the most abundant class of hydrocarbons, and their profiles differed dramatically between sexes. In males, the 23-carbon alkene accounted for about half of the total hydrocarbon. In females, this alkene was far less

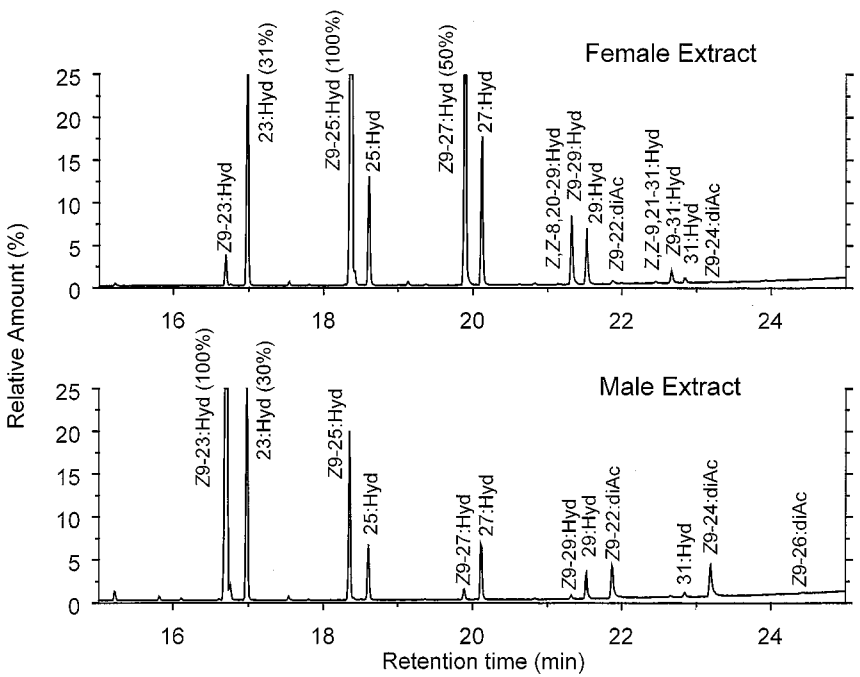


FIG. 2. Gas chromatograms of extracts from female and male *C. cinctus*. Selected hydrocarbons (:Hyd) and diacetates (:diAc) are labeled. Relative areas of off-scale peaks are shown in parentheses.

TABLE 2. HYDROCARBONS EXTRACTED FROM 2-TO 5-DAY-OLD MALE AND FEMALE *C. cinctus* CUTICLE

Hydrocarbon	Relative amount (% of total)		MS data: molecular ion of hydrocarbons (and key ions of DMDS adducts)
	Females	Males	
n-Alkanes (by numbers of carbons)			
21	0.31	0.57	296
22	0.13	0.29	310
23	13.9	17.5	324
24	0.24	0.23	338
25	8.6	6.1	352
26	0.32	0.20	366
27	11.1	5.9	380
28	0.20	0.14	394
29	3.5	3.4	408
30	0.038	0.074	422
31	0.52	1.2	436
33	0.10	0.36	464
n-Alkenes (by double bond descriptions and numbers of carbons)			
(*Z*)-9-21	0.016	0.24	294, (173, 215, 388)
(*Z*)-9-22	0.027	0.48	308, (173, 229, 402)
(*Z*)-9-23, [(*Z*)-7-23][a]	4.4	48.0	322, (173, 243, 416, [145, 271][a])
(*Z*)-9-24	0.53	0.68	336, (173, 257, 430)
(*Z*)-9-25	34.9	13.4	350, (173, 271, 444)
(*Z*)-9-26	0.50	0.072	364, (173, 285, 458)
(*Z*)-9-27	16.6	0.92	378, (173, 299, 472)
(*Z*)-9-28	0.15	ND[b]	392, (173, 313, 486)
(*Z*)-9-29	3.1	0.17	406, (173, 327, 500)
(*Z*)-9-30	0.049	ND	420, (173, 341, 514)
(*Z*)-9-31	0.67	0.071	434, (173, 355, 528)
n-Alkadienes (by double bond descriptions and numbers of carbons)			
(*Z*,*Z*)-8,20-29	0.034	0.020	404, (159, 173, 325, 339, 498, 592)
(*Z*,*Z*)-9,21-31	0.042	0.015	432, (173, 187, 339, 353, 526, 620)

[a] Present in trace amount (<1% of major isomer).
[b] ND denotes not detected.

abundant, and the 25- and 27-carbon alkenes were dominant instead. There were smaller amounts of alkenes with 21, 29, and 31 carbons in both sexes and also the intervening even-chain compounds. In both sexes, the double bonds were almost exclusively in the nine position for all chain lengths, based on mass spectra of the DMDS derivatives. The 23-carbon alkenes from both sexes did have trace amounts of 7-alkene (<1% as much as the 9-isomer). The observed molecular ions (alkene molecular weights plus 94) and the key fragment ions (from cleavage between the carbons with the thiomethyl groups) are given in Table 2. By comparison to *E* and *Z* synthetic standards (underivatized) on the $AgNO_3$ HPLC

column, all of the sawfly alkenes had the *Z* configuration. No branched alkenes were detected.

In females, hydrocarbon mass spectra indicated the presence of small amounts of alkadienes of 29 and 31 carbons (molecular ions at $m/z = 404$ and 432, respectively). The mass spectra of the DMDS derivatives were interpreted according to Carlson et al. (1989). Fragments listed in Table 2 represent the molecular ion (addition of two equivalents of DMDS), the loss of 94 (one DMDS) as a neutral molecule, and subsequent cleavage at the remaining DMDS site as with the alkenes (yielding four ions, representing the saturated and unsaturated side of each double bond). These spectra determined the double bonds to be in the 9 and 21 positions from one end of the chain for both dienes. (However, rules of nomenclature require that the 29-carbon compound be named as 8,20-nonacosadiene so that the locant numbers are as small as possible). GC retention times were consistent with straight chains and *Z* configurations at the double bonds (Bartelt et al., 1986). These dienes were also detected from males, but in smaller amounts.

The total amount of hydrocarbon per sawfly was about 15 μg, with males and females having very similar quantities. Extraction for 20 min or 24 hr gave comparable profiles and amounts, although the longer soak also extracted fatty acids (primarily palmitic and oleic) and sterols from the insects. Even a 1 sec rinse gave essentially the same profiles, indicating that the compounds were present on the immediate surface. The lipid profiles and amounts were consistent in major features for all ages between 1 hr and 5 days. In contrast, adults removed from wheat stubs before natural emergence had the typical amount of alkane but only about 10% of the usual amount of alkene. Apparently, biosynthesis of alkenes is intense around the time of emergence. Finally, younger females (several hours to 1 day old) had larger amounts of the alkadienes than older ones, with the 29-carbon diene amounting to as much as 0.5% of the total hydrocarbon rather than less than 0.05%. This difference was not seen in males. The female in Figure 2 was less than 1 day old at the time of extraction.

Diacetate Lipids. Initial comparison of chromatograms revealed two compounds in male extracts (approximately 1 μg of each per male) that were far less abundant in females (Figure 2, retention times of 21.9 and 23.2 min). The mass spectrum of the first of these indicated a molecular weight of 424 (Figure 3), which was not consistent with a hydrocarbon. (A fully saturated hydrocarbon of 30 carbons would have a molecular weight of 422; thus oxygen was probably present in the sawfly compound to account for the higher molecular weight). Several subtle features in the mass spectrum gave important information about the structure. The first was a fragment at m/z 61 (19% of base peak), which is characteristic for an acetate group (McLafferty, 1980). Examination of library spectra of acetates indicated that the m/z 61 peak is most intense when the acetate group is at or near the end of the carbon chain and is distant from other functional groups or branches. Second, the spectrum of the sawfly compound lacked the characteristic peaks that

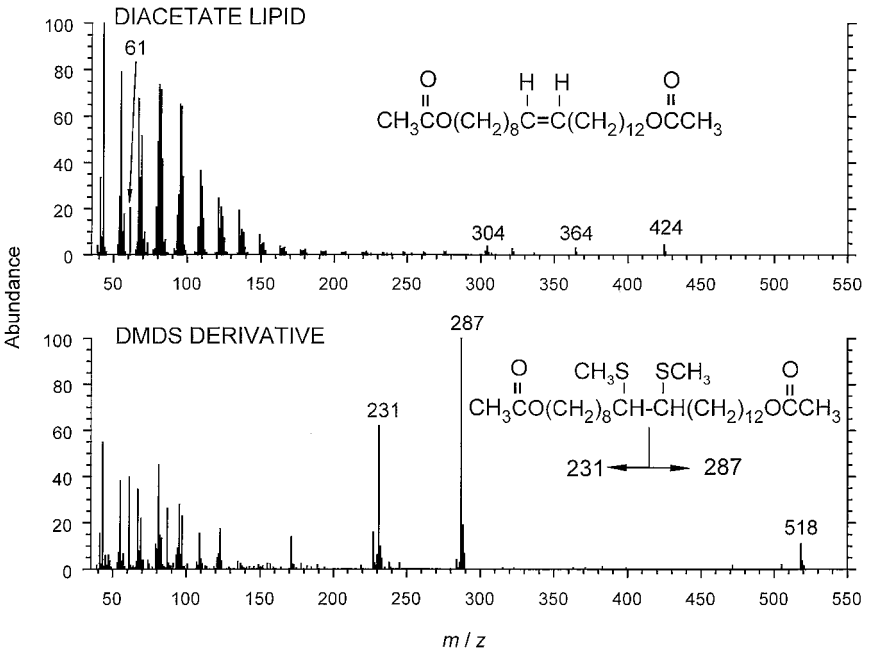


FIG. 3. EI-mass spectrum of 22-carbon diacetate lipid from male *C. cinctus* (top) and EI-mass spectrum of the DMDS derivative of this lipid, showing origins of major fragments (bottom).

would be expected if the acetates were secondary; for example, an acetate on the second carbon of a chain leads to a prominant spectral peak at *m*/*z* 87 due to chain cleavage adjacent to the acetate. Finally, there were fragments at *m*/*z* 364 and *m*/*z* 304 that corresponded to two consecutive losses of 60 from the molecular ion (believed to represent the loss of two acetic acid molecules). Taken together, this evidence suggested a lipid chain of 22 carbons with an acetate group on each end. However, the molecular weight for a saturated chain would be 426 instead of 424; thus a double bond was probably present in the chain. The DMDS derivative formed readily (molecular ion at *m*/*z* 518 = 424 + 94), and the fragment ions (*m*/*z* 231 and 287) were consistent with a double bond being present in the 9 position of the original lipid (Figure 3).

The data for the second lipid were analogous, with molecular weight of 452 and key fragments at *m*/*z* 61, 332, and 392. The DMDS adduct gave key MS ions at *m*/*z* 231, 315, and 546, consistent with a 24-carbon diacetate, again with the double bond in the 9 position. Closer inspection of the gas chromatograms revealed a third, still later-eluting member of this series, about 10% as abundant as the first two. Its mass spectrum included ions at *m*/*z* 61, 360, 420, and 480, and the MS

ions of its DMDS derivative included 231, 343, and 574. These data indicated a 26-carbon diacetate, once more with the double bond in the 9 position. There was no evidence for other double-bond positional isomers. Close examination of female-derived extracts and their DMDS derivatives by GC-MS revealed that the same three compounds were present but at much lower levels (about 10% of the amounts in males, see Figure 2).

Synthetic Alkenediol Diacetates. Synthetic alkenediol diacetates were prepared to confirm the proposed structures and to establish double-bond configuration of the natural material. The mass spectral data (Table 1) supported that the synthetic reactions proceeded as shown in Figure 1 because the synthetic intermediates and products had the expected molecular weights and reasonable fragmentation patterns. For example, THP ethers were readily recognized by their mass spectra (Table 1) because of the base peak at m/z 85 and a moderately intense ion at m/z 101, due to cleavage on either side of the ether oxygen. The molecular ion and an M-1 ion were usually seen for the THP ethers, but the intensity for both was low (ordinarily $<1\%$). The M-85 peak was similarly small but was still reliably present and served as further confirmation of molecular weight. Example details for one THP ether alcohol and one THP ether aldehyde are given under the intermediates for compound **5** in Table 1; for other THP ethers, only key ions are listed. Analogously to THP ethers, benzyl ethers had spectral peaks at m/z 91 and 107. The THP ether bromides (intermediates for **1** and **2**) had characteristic spectra because of the bromine isotopes. For the di-THP diether precursor of **8**, the molecular ion was not seen, but the M-85 fragment was present, and there was another ion corresponding to two losses of a deprotonated THP group (m/z 340). After deprotection, the free diol exhibited a small molecular ion and ions corresponding to one and two neutral losses of water. Features of the mass spectra of alkenediol diacetates were discussed above.

Patterns in GC retention times supported the mass spectral interpretations and synthetic expectations. For example, the Wittig reactions always gave two products (the *E* and *Z* isomers) that were separated by about 0.1 min but had nearly identical mass spectra. Catalytic hydrogenation always collapsed the E/Z peak doublet into a single peak of still higher GC retention time. Also, oxidation of a THP ether alcohol to the corresponding THP ether aldehyde consistently decreased the GC retention time (Table 1).

By GC of synthetic **8–10**, the earlier-eluting isomer was always the more abundant one; this was expected to be the *Z* isomer because the Wittig reaction conditions favor production of *Z* double bonds (Sonnet, 1974). Compound **10** was analyzed further by AgNO3 HPLC to confirm this. The more abundant isomer eluted later than the less abundant one (24–26 ml vs. 20–21 ml, based on GC analysis of HPLC fractions), which also supported that the more abundant isomer was *Z* (Heath and Sonnet, 1980). The IR spectra further corroborated this conclusion. The more abundant isomer was a liquid at room temperature and had key IR

absorbances (cm^{-1}) indicative of an olefin (3003, w, =C–H), hydrocarbon chain (2925 and 2854, both s, $-CH_2-$), and acetate group (1741, s, C=O; 1365, m, 1240, s, and 1037, m, other acetate-related peaks), but the spectrum did not indicate presence of an *E* double bond. On the other hand, the less abundant isomer was a solid at room temperature and had corresponding absorbances at 2918, 2850, 1732, 1369, 1247, and 1041; it also had the absorbance (962, m) that confirmed the *E* double bond. The proton NMR spectrum of the more abundant isomer supported the structure for synthetic **10** as drawn in Figure 1, but it gave no further information about double-bond configuration because the olefinic protons had the same shift and the coupling constant could not be observed: δ 5.56 (2 H, m, =C$\underline{H}$–), 4.04 (4 H, m, O–C$\underline{H}_2$–), 2.17 (4 H, m, =CH–C$\underline{H}_2$), 1.74 (6 H, s, C$\underline{H}_3$(C=O)O), 1.2–1.6 (40 H, other –C$\underline{H}_2$–, m).

Comparison of Natural and Synthetic Alkenediol Diacetates. Based on same-day comparisons of GC retention times, each natural alkenediol diacetate matched exactly the *Z* isomer of the corresponding synthetic compound for all three chain lengths. They also matched with respect to mass spectra and with respect to GC rentention times and mass spectra of the DMDS derivatives.

Compounds in Nature Related to Diacetates. This is believed to be the first report of natural alkenediol diacetates, but related compounds are known. Beeswax contains saturated 1,ω-diols of 24–28 carbons and esters of these, but the acid moieties have 16–24 carbons instead of just two (Tulloch, 1971). Free 1,ω-alkanediols of 22, 24, and 26 carbons have been isolated from various plants (Chatterjee and Chakraborty, 1967; Austin et al., 1969; Rizvi and Sultana, 1972; Amparo-Lopez et al., 1976; Hatam et al., 1990). 1,16-Hexadecanediol diacetate has been reported in the defensive secretion of the gulf fritillary butterfly (M. Blum, personal communication). Recently Oliver et al. (2000) and Doss et al. (2000) isolated 3-hydroxypropanoyl esters of (*Z*)-9-docosene-1,22-diol and (*Z*)-9-tetracosene-1,24-diol from bruchid weevils. The compounds are potent plant growth regulators, and callus tissue develops on legume pods exposed to these compounds during oviposition by female beetles.

Oxidation of Cuticular Alkenes. "Spontaneous" oxidation of unsaturated lipids has been studied extensively, especially in relation to rancidity of food oils, and much is known about the chemical mechanisms involved (reviewed by Frankel, 1998). Oxidation is complicated and involves cascades of free-radical reactions. It can be light induced (photooxidation) or simply occur by exposure to molecular oxygen under ambient conditions (autoxidation) if triggered by trace catalysts or reactive species such as free radicals. Once initiated, the reactions tend to be self-sustaining. Both mechanisms generally lead to the same volatile products, but proportions can differ. The major volatile products are usually aldehydes.

One food-related lipid, methyl oleate, provides a reasonable predictive model for the nonenzymatic oxidation of the alkenes and cuticular alkenediol diacetates of *C. cinctus*. Methyl oleate is like the sawfly compounds in having just one *Z* double

bond that is distant from other functional groups or chain ends. The mechanism for oxidation of methyl oleate to volatile products (Frankel, 1998) suggests a specific series of compounds that would result from the oxidation of particular alkenes, and occurrence of these compounds in the volatile collections from sawflies would support that such a mechanism is operating.

The oxidation scheme and expected products from (*Z*)-9-tricosene, the only abundant alkene in *C. cinctus* males, are summarized in Figure 4. The initial reaction is the formation of allylic hydroperoxides (four positional isomers). The hydroperoxides decompose either thermally or catalytically (e.g., by metal ions) into alkoxyl radicals, and these can then react in a number of ways, including cleavage into volatile products. Scission on either side of the radical group (indicated by "A" or "B" in Figure 4), yields a specific free aldehyde plus an intermediate that reacts further to give a characteristic hydrocarbon, alcohol, or other aldehyde. This general scheme is shown at the bottom of Figure 4, and the expected products from each of the hydroperoxide isomers are summarized at the upper right. By analogy to methyl oleate (Frankel, 1998), nonanal and tetradecanal should be the most abundant products.

The compounds actually detected in volatile collections are shown in bold print in Figure 4; relatively large and intermediate quantities are indicated by ** and *, respectively. The compounds were identified by mass spectrum and GC retention time, relative to authentic standards. The more abundant products are also indicated in the total ion chromatogram in Figure 5, which represents a volatile collection from male sawflies.

Nonanal and tetradecanal were, in fact, the most abundant of the expected oxidation products from males. Octanal and decanal were clearly present but were less abundant. Amounts were still smaller for the other compounds noted in bold in Figure 4. Some of the products expected but not found (heptanal, heptane, heptanol, octane) are relatively volatile and would have occurred too near the solvent front of the GC run to yield sharp peaks and be easily detected. Others, such as 2-undecenal, were probably present, but small amounts and coelution with more abundant compounds prevented positive identification. The heavier (15- and 16-carbon) 2-alkenals were not detected. Their lower volatilities would have reduced the amounts collected. Furthermore, these compounds are especially prone to further oxidation, and remaining in the oxidizing lipid mixture instead of evaporating out would have enhanced their degradation.

Female sawflies have very little 23-carbon alkene but have large amounts of the 25- and 27-carbon alkenes. Oxidation of these would be analogous to tricosene. All of the scission products with 11 or fewer carbons (Figure 4) would be expected from any of the 9-alkenes, but those of 12 or more carbons expected from 9-tricosene would be replaced by homologs with two or four additional carbons if the parent alkene is 9-pentacosene or 9-heptacosene. Hence, there was relatively little tetradecanal from females, but hexadecanal was abundant instead (Cossé

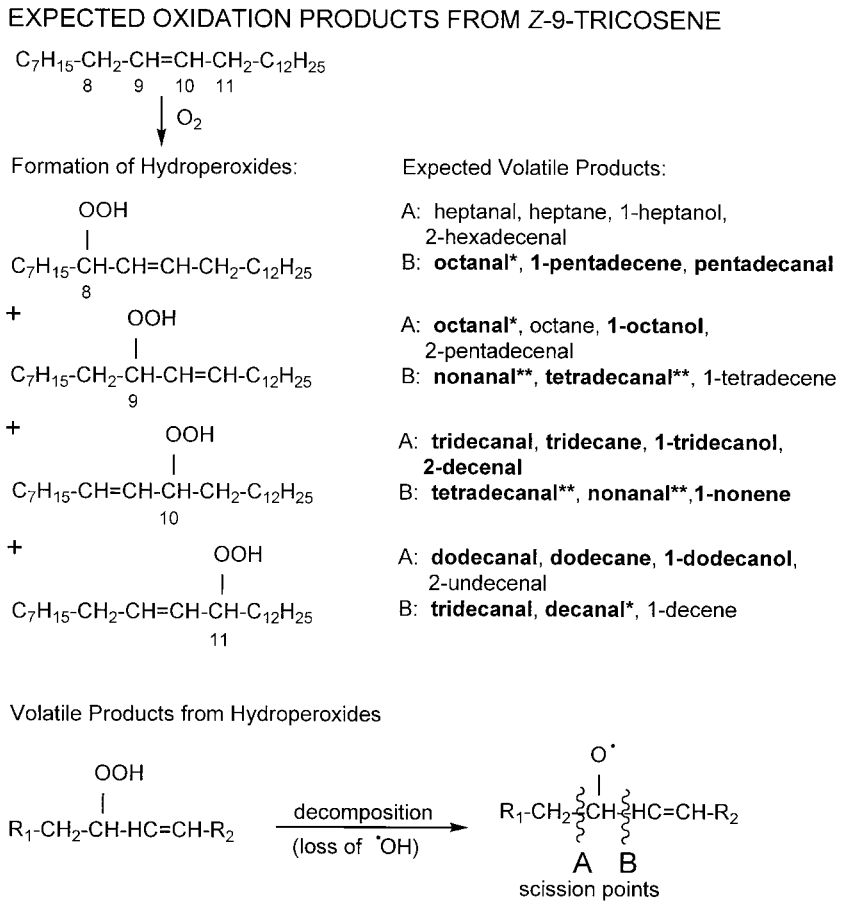


FIG. 4. Expected products from the autoxidation of (*Z*)-9-tricosene, based on the process for methyl oleate (Frankel, 1998). Compounds in bold print were detected in volatile collections from male sawflies. High and intermediate relative abundance denoted by ** and *, respectively.

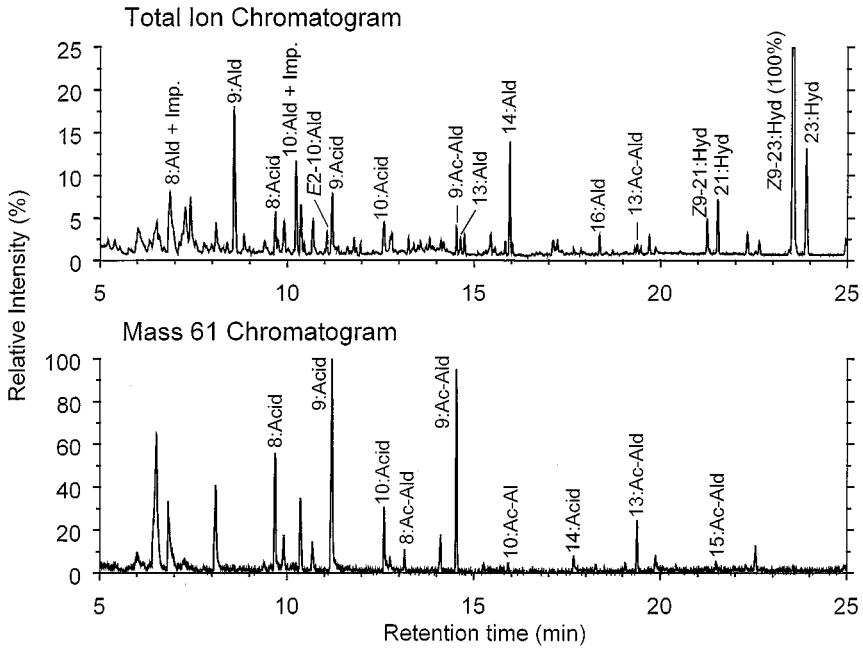


FIG. 5. GC-MS chromatograms of a volatile collection from male *C. cinctus*. The upper trace is total ion chromatogram. The lower trace is for mass 61 only, which shows the acetyloxyaldehydes (and also the carboxylic acids) very clearly. Peaks are labeled as aldehydes (:Ald), hydrocarbons (:Hyd), carboxylic acids (:Acid), or acetyloxyaldehydes (:Ac-Ald). Nonbiological impurities are denoted by "Imp."

et al., 2002). The 18-carbon aldehyde was observed in the collections of volatiles but in smaller amounts. Its lower volatility probably reduced its abundance in the collections.

The observed product patterns support the formation of the volatiles by a mechanism like that described for methyl oleate. Further oxidation of these aldehydes would yield carboxylic acids, and those of 8, 9, and 10 carbons were found consistently in the volatile collections from sawflies (Cossé et al., 2002) (Figure 5), although these may have other, as yet unexplained sources as well. The emission of phenylacetic acid from sawflies is not, however, accounted for by the oxidation mechanism.

The amounts of oxidation products collected from the sawflies were minute relative to the amounts of alkene present. The most abundant aldehydes from males were nonanal and tetradecanal, but typical collected amounts were only 50 and 15 ng/day per male, respectively, while males have about 7 μg of the parent 9-tricosene. Monounsaturated compounds such as methyl oleate oxidize

only 1–2% as rapidly as the polyunsaturated analogs (Frankel, 1998); thus, the minor yields of volatile products from alkenes is not surprising. Nevertheless, the levels of volatiles observed are still sufficiently high to be detectable to sawfly antennae. Intriguingly, even though the intact alkenes were not found to elicit electrophysiological responses (Cossé et al., 2002), the male–female dimorphism in alkenes could still lead to behavioral recognition of sexes at a distance by way of the aldehyde (or other) oxidation products.

Dienes. The alkadienes, which are more abundant in females, would release additional, unique oxidation products. These would include dodecanedial and unsaturated aldehydes of 20, 21, and 22 carbons with a double bond in the 12 position. None of these compounds were detected, but the amount of diene starting material that was present was extremely small.

Oxidation of Alkene-1,ω-diol Diacetates. By analogy to the alkenes, the alkenediol diacetates of 22, 24, and 26 carbons would all be expected to produce 9-acetyloxynonanal, and each diester would also generate a unique, heavier homolog from the other end of the lipid chain (Figure 6).

The mass spectral data files for collections of sawfly volatiles were examined for these compounds by extracting single-mass chromatograms for *m*/*z* 61 (for

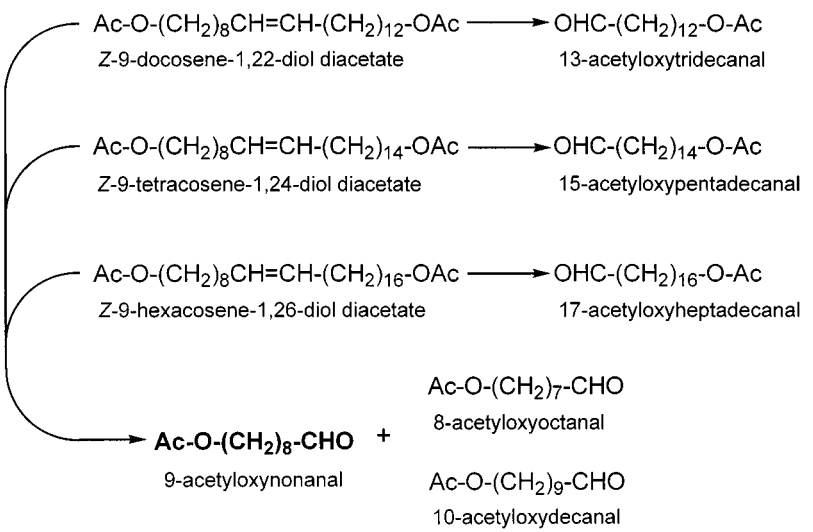


FIG. 6. Acetyloxyaldehydes expected from the oxidative cleavage of the alkenediol diacetates of *C. cinctus*. The major product would be 9-acetyloxynonanal because it can be derived from all three parent lipids, and acetyloxyaldehydes with 13-, 15-, and 17-carbon chains would result from the other ends of the parent lipid chains. Analogs of these acetyloxyaldehydes having one additional or one fewer carbon would also be expected (see text), but only those related to 9-acetyloxynonanal were likely to be detectable.

example, Figure 5). This approach revealed compounds containing the primary acetate group without interference from many other functional classes. (Simple aldehydes, alcohols, and hydrocarbons do not exhibit the *m*/*z* 61 fragment. Organic acids do show up, however, because there is always an isotope peak or protonated fragment corresponding to the major ion at *m*/*z* 60). 13-Acetyloxytridecanal was readily found in samples in which 9-acetyloxynonanal was prominent (about 20% as abundant as the major product, Figure 5). The 15-carbon homolog was sometimes detectable but was even less abundant, probably because of lower volatility. 17-Acetyloxyheptadecanal was never found, presumably because of still lower volatility and the much smaller amount of precursor. All four of these aldehyde acetates (of 9, 13, 15, and 17 carbons) were detected when the crude hexane extract of sawflies was ozonized, and the 13-carbon homolog clearly stimulated antennae (Cossé et al., 2002).

8-Acetyloxyoctanal and 10-acetyloxydecanal were also detected in volatiles collections but not from the lipid ozonolysis experiment. These compounds would be expected if the alkenediol diacetates oxidized according to the scheme for the alkenes and methyl oleate (but not from ozonolysis). Thus the pattern of aldehyde acetates observed in volatiles collections further supported the idea that they were formed by "weathering" of cuticular lipids. Interestingly, in tests with the synthetic standards, 8-acetyloxyoctanal and 10-acetyloxydecanal were clearly active by GC-EAD. Analogs with one additional and one fewer carbon would also be expected for the acetyloxyaldehydes with 13-, 15-, and 17-carbon chains, but these would have been present at extremely low levels and were not detected.

Under laboratory conditions, the amount of 9-acetyloxynonanal emitted from male sawflies was small (1–10 ng per sawfly per day) (Cossé et al., 2002) relative to the amounts of precursor alkenediol diacetates present in males (about 2 μg), but it was roughly proportional to the amounts of aldehydes apparently derived from 9-tricosene, further supporting a similar origin. In addition, the relatively greater production of 9-acetyloxynonanal from male sawflies than females (Cossé et al., 2002) was consistent with the greater amounts of the alkenediol diacetates present in males.

These qualitative and quantitative relationships strongly suggest that the alkenediol diacetates are the precursors to 9-acetyloxynonanal, a key compound in the sawfly volatiles collections that elicited intense antennal activity and had demonstrable activity in field tests (Cossé et al., 2002), and furthermore, that passive oxidation of the cuticular lipids could account for the amounts obtained in our volatiles collections from living sawflies.

Laboratory Oxidation of Alkenediol Diacetates. The synthetic alkenediol diacetate **10** was free of detectable acetyloxyaldehydes prior to artificial sunlight exposure. However, after 2 hr of irradiation, and as expected from our chemical model (Figure 6), 8-acetyloxyoctanal, 9-acetyloxynonanal, 16-acetyloxyhexadecanal, and 17-acetyloxyheptadecanal could be readily detected and were 0.09%, 0.4%,

0.3%, and 0.9% as abundant as the recovered starting material **10** (calculated from peak areas in the total ion chromatogram). 10-Acetyloxydecanal and 18-acetyloxyoctadecanal were not detected. An HPLC preparation of natural alkenediol diacetates, which contained **8** and **9** in an 8:1 ratio, was also free of detectable acetyloxyaldehydes before the light treatment. After 2 hr of light exposure, the results were analogous to synthetic **10:** 8-acetyloxyoctanal, 9-acetyloxynonanal, 12-acetyloxydodecanal, 13-acetyloxytridecanal, and 15-acetyloxypentadecanal were detected and were, respectively, 0.1%, 0.6%, 0.2%, 0.8%, and 0.2% as abundant as starting compound **8**. The experiment with synthetic **10** without exposure to light gave results similar to when the light was used, but the amounts of the acetyloxyaldehydes were lower (the acetyloxyaldehydes with 8-, 9-, 16-, and 17-carbon chains were 0.03%, 0.2%, 0.1%, and 0.4% as abundant as the starting material, respectively).

Although this experiment was preliminary, it clearly demonstrated that the biologically active acetyloxyaldehydes can be produced from the alkenediol diacetates in the absence of living sawflies. Furthermore, the amounts of acetyloxyaldehydes produced, relative to the amounts of parent alkenediol diacetates, were remarkably similar to those encountered with living sawflies (e.g., low nanogram quantities of acetyloxyaldehydes emitted per sawfly per day versus low microgram quantities of alkenediol diacetate present on the cuticle). Based on the GC-EAD studies of Cossé et al. (2002), the amounts of acetyloxyaldehydes produced by this process are large enough to be biologically relevant.

Behavioral and Environmental Enhancement of Oxidation Reactions. Certain features of sawfly biology would favor the oxidation of cuticular lipids. The insects are most active when the weather is warm (microclimate as hot as 35°C) and sunny (D. K. Weaver, unpublished). The oxidation reactions are accelerated by both heat and sunlight (Frankel, 1998, and our preliminary experiment). Sawfly grooming behavior (D. K. Weaver, unpublished) could distribute pheromonal precursors over the cuticle in a way that enhances oxidation rates. Thus it is possible that the reactions may be biologically (behaviorally) controlled even if there is no direct enzymatic mediation. The abiotic oxidation of unsaturates is complicated to study, especially in the early stages of the process, because it can be triggered or influenced by so many factors. These include the presence of trace amounts of metal ions, ketones, hydroperoxides, and other reaction-initiating substances, the action of ultraviolet light, and the effect of temperature (Frankel, 1998). On the other hand, involvement of enzymes has not been ruled out, and resolving this issue will be essential to understanding this pheromone system.

Pheromones Produced by Oxidation. The oxidation of relatively nonvolatile cuticular lipids is known to release volatiles with pheromonal activity in other hymenopterans. For example, females of the yellowheaded spruce sawfly, *P. alaskensis*, possess a series of (*Z*,*Z*)-9,19-alkadienes of 28–37 carbons that is absent in males (Bartelt et al., 1982). All of these dienes have a common oxidation

product, (*Z*)-10-nonadecenal, that is highly attractive to males in the field (Bartelt and Jones, 1983). Similarly, in the parasitic wasp, *Macrocentrus grandii* Goidanich (Braconidae), the females have a series of (*Z*,*Z*)-9,13-alkadienes of 27–41 carbons that the males lack (Swedenborg and Jones, 1992). With these dienes, the common oxidation product is (*Z*)-4-tridecanal, and as with *P. alaskensis*, the aldehyde is clearly attractive to males in the field. In laboratory wind-tunnel bioassays, as little as 17 pg of this aldehyde elicited dramatic upwind orientation.

C. cinctus differs in two important ways from the above examples. First and most obviously, the males are the major source of the key compounds (e.g., the diacetate lipids and the derived aldehyde acetates) instead of the females. Second, *both* sexes do have measurable amounts of the compounds, unlike the usual pattern with insect sex pheromones. Although the pheromone research on *C. cinctus* is in its early stages, the pheromone biology of the species already appears to be quite atypical and may provide a useful model for comparison in the future.

The existence of oxidation-derived pheromones in insects has not been frequently explored. The distinctive alkenediol diacetates of *C. cinctus* and their chemical relationship to the acetyloxyaldehydes through oxidation made this connection between nonvolatile precursors and volatile pheromones very clear, but as demonstrated with the alkenes, very typical compounds can also release volatiles that could be every bit as important biologically as the more esoteric ones. The reported examples of biologically active oxidation products are from the Hymenoptera, but it seems likely that the phenomenon could occur far more widely.

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PHEROMONE COMPONENTS OF THE WHEAT STEM SAWFLY: IDENTIFICATION, ELECTROPHYSIOLOGY, AND FIELD BIOASSAY[1]

ALLARD A. COSSÉ,[2,]* ROBERT J. BARTELT,[2] DAVID K. WEAVER,[3] and BRUCE W. ZILKOWSKI[2]

[2]*USDA Agricultural Research Service*
National Center for Agricultural Utilization Research
Crop Bioprotection Research Unit
1815 N. University Street
Peoria, Illinois 61604

[3]*Montana State University*
Department of Entomology
333 Leon Johnson Hall, P.O. Box 173020
Bozeman, Montana 59717-3020

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Abstract—Volatiles collections and cuticular extracts of the wheat stem sawfly, *Cephus cinctus* Norton (Hymenoptera: Cephidae), were analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD) in order to detect possible pheromone components of this species. Volatiles collections from male and female sawflies contained the same 13 GC-EAD-active compounds that stimulated both male and female antennae. GC-EAD-active compounds were identified by mass spectrometry, and the identities were verified by acquiring or synthesizing authentic standards with identical GC retention times and mass spectra. 9-Acetyloxynonanal gave the strongest EAD response. Additional GC-EAD-active compounds included 13-acetyloxytridecanal, aldehydes with 9–16 carbon chain lengths, acids with 8–10 carbon chain lengths, and phenylacetic acid. The last was instantly detectable by human nose when several males were allowed to interact, but not from isolated insects. On a per-insect basis, grouped males overall released higher amounts of the GC-EAD-active compounds than grouped females. Notable quantitative differences between the sexes were relatively higher amounts of 9-acetyloxynonanal, phenylacetic acid, and tetradecanal from males and hexadecanal from females. In the absence of an absolute, qualitative difference between sexes, these relative differences might be im-

*To whom correspondence should be addressed. E-mail: cosseaa@ncaur.usda.gov

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portant for sex recognition. Field bioassay demonstrated the attractiveness of 9-acetyloxynonanal for male and female *C. cinctus*. The major compounds of male and female cuticular extracts showed no GC-EAD activity, but ozonolysis of extracts gave dramatically increased amounts of GC-EAD-active material. Hence, double-bond-containing cuticular compounds are suggested as precursors of the aldehydic pheromone components.

Key Words—Sawfly, aggregation pheromone, 9-acetyloxynonanal, phenylacetic acid, gas chromatographic–electroantennographic detection, cuticular extracts, *Cephus cinctus*, Cephidae, Hymenoptera.

INTRODUCTION

The wheat stem sawfly, *Cephus cinctus* Norton (Hymenoptera: Cephidae), has been a major pest of wheat, *Triticum aestivum* L, in the northern Great Plains of the United States and the Canadian prairies since cultivation began (Weiss and Morrill, 1992). In Montana, the insect has been estimated to cause annual losses in excess of $25 million in recent years (Blodgett et al., 1996, 1997), and losses in the entire area impacted by the wheat stem sawfly are estimated to exceed $100 million (US Department of Agriculture, 2000). The biology of the insect is described in Ainslie (1920), and population dynamics are given in Holmes (1982). Briefly, short-lived adults emerge from wheat stubble in the spring and fly to cereals or other grasses, where they oviposit into the interior of growing stems. The larvae feed and develop cryptically within the stem during the summer. When crop maturity is reached and the host plant desiccates, the larvae descend to the base of the plant and girdle the stem near the soil interface, resulting in lodged plants. The mature larva seals the tip of the cut stem with a uniform plug and then descends to near the root where it overwinters as a mature larva within a translucent hibernaculum. Dispause is broken in the spring, and metamorphosis is completed. Adults chew a hole in the plug at the tip of the cut stem and move to the surrounding green vegetation.

The female-produced sex pheromones of sawflies belonging to the family Diprionidae and one species belonging to the family Tenthredinidae are well documented (Anderbrant 1999 and references therein). In addition, studies on several species belonging to the family Pamphiliidae have shown that female-produced sex pheromones are used for mate location (Borden et al., 1978). However, no studies have been published on pheromones in the remaining 11 families of hymenopteran sawflies, including Cephidae. Understanding the chemical ecology of *C. cinctus* could lead to new and environmentally friendly approaches for reducing agricultural damage, especially since insecticides are ineffective against larvae inside the wheat stem and adult emergence is difficult to predict (Morrill et al., 1992).

This paper and the companion paper (Bartelt et al., 2002) describe the identification, electrophysiological, chemical, and behavioral aspects of pheromonal communication in the wheat stem sawfly.

METHODS AND MATERIALS

Insects. Overwintering stage prepupae of *C. cinctus* were collected at several field sites in Montana. After the 1998 harvest, collections were made in September and October in Broadwater and Cascade counties, and again in March and April, 1999, from the same sites. Stubble was removed from these heavily infested fields with a shovel and taken to the laboratory. The uninfested residue was separated from the sawfly-cut stems, and stems containing sawfly larvae were rinsed to remove the soil, spotted dry with a paper towel, and stored in lots of 100 in plastic Ziploc bags. To allow the larvae to complete diapause, bags containing fall-collected larvae were stored at 4°C for at least 100 days, while material collected in late winter and early spring was refrigerated until used. Upon arrival in Peoria, Illinois, sawfly-containing wheat stems were placed individually into glass vials capped with a moist piece of cloth-covered cotton. Adults emerged at 25°C under a 16L:8D photoperiod regime using a fluorescent light source.

Initial Cuticular Extracts. Groups of 25 male and 25 female sawflies were killed by freezing when 1–3 days old and cuticular extracts were obtained by soaking insects for 20 min in 1 ml hexane. Hexane extracts were stored at −20°C.

Volatiles Collections. Volatiles collections were made from individuals or groups of two to five male or female insects. The 1- to 3-day-old adults were placed in 75-ml silanized (dichlorodimethylsilane) (Bartelt and Zilkowski, 1998) Pyrex glass tubes fitted with corks at both ends, into which Super Q porous polymer (80–100 mesh, Alltech, Deerfield, Illinois) traps were inserted. The Super Q traps were prepared from 10-cm × 0.5-cm-ID glass tubing. A piece of stainless steel screen (325 mesh) was sealed into one end by heating. Traps were filled to a depth of 0.5 cm with Super Q and topped with a plug of silanized glass wool. Newly prepared and used traps were conditioned by several rinses of CH_2Cl_2 followed by hexane and dried under a gentle stream of nitrogen. Traps used to collect volatiles from male and female insects were kept separated. Insects had access to water from 2 ml cotton plugged vials, which were placed inside the collection tubes. Volatiles collections from tubes with water vials, but without insects, served as controls. A vacuum was applied to one of the Super Q traps so that volatiles from the tube were drawn into the trap. The second Super Q trap then served as a filter for incoming air. The tubes were kept under fluorescent light in an incubator (16L:8D) at 27°C and 40% relative humidity with an airflow through the tubes adjusted to 30 ml/min. Two- to three-day volatiles collections were started 2–3 hr into the photophase. To extract volatiles from the Super Q traps, each trap was rinsed with 250 μl hexane. Samples were concentrated to approx. 10 μl under a gentle stream of nitrogen and 1 μl was analyzed. Approximately 160 volatiles collections were made.

Solid-phase microextraction (SPME) was used as a second volatiles collection method. Individuals and groups of up to five adults were placed in the above-mentioned silanized Pyrex glass tubes. The upwind end of a glass tube was

covered with nylon mesh and the downwind end was fitted with a cork, into which a glass tube (0.4 cm ID) with a side arm was inserted. A SPME fiber (100 μm polydimethylsiloxane. Supelco) was inserted though a septum placed into the other end of the tube, extending past the side arm junction. Vacuum was applied to the side arm so that air from the outside passed though the tube with the sawflies and then over the SPME fiber. Volatiles collections were made 2–3 hr into the photophase at 25°C for 30 min with airflow of 10 ml/min. Insects could not contact the SPME fiber.

Electrophysiology. Coupled gas chromatographic–electroantennographic (GC-EAG) analyses were made by methods and equipment generally described by Cossé and Bartelt (2000).

Pheromone Identification. Volatiles collections were analyzed by gas chromatography with flame ionization detection (GC-FID), coupled GC-mass spectrometry (GC-MS), and GC-EAD. The GC was a Hewlett Packard 6890 instrument fitted with a 30-m EC-1 capillary column (0.25 mm ID, 0.25 μm film thickness, Alltech). Temperature programs were from 50°C to 275°C at 10°C/min. (GC-FID) or from 50°C to 275°C at 15°C/min. (GC-EAD). Injector temperatures were maintained at 250°C, and the GC-EAD effluent interface from the post-colum splitter was kept at 275°C. Injectors were fitted with SPME injector liners (Supelco, Bellefonte, Pennsylvania) for SPME analysis. Mass spectrometry was performed by using a Hewlett Packard 5973 instrument (electron impact, 70 eV). Chemical ionization spectra (methane) were obtained on a Finnigan 4535 quadrupole mass spectrometer with GC inlet. Most EAD-active compounds were identified by searches of the mass spectral library (Wiley, 1995). Identifications were confirmed with authentic standards that had the same GC retention times and mass spectra.

Ozonolysis of cuticular extracts was done as described by Beroza and Bierl (1967), and the products were compared with the compounds in the volatiles collections. Samples were ozonized in 0.5 ml dichloromethane for about 2 min at −70°C. Ozone was formed by electrical discharge through a stream of oxygen. The gas was bubbled gently through the solution until a faint bluish color developed, indicative of excess ozone. Ozonides were reduced with excess triphenylphosphine. Samples were analyzed by GC-MS and GC-EAD.

Chemicals. Nonanal and (*Z*)-9-dodecen-1-ol acetate were purchased from Bedoukian (Danbury, Connecticut), and the remaining commercially available chemicals used in this study were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin).

Synthesis of 9-Acetyloxynonanal. A solution of (*Z*)-9-dodecen-1-ol acetate (100 mg, 0.44 mmol) in 5 ml hexane was cooled in a dry ice–ethanol bath. Ozone was bubbled through the solution until a faint bluish color developed, indicative of excess ozone. The solution was purged with N_2 until the blue color disappeared. The reaction mixture was reduced by adding dimethylsulfide (0.66 mmol) and allowed to warm to room temperature. After addition of 0.5 ml H_2O and separation

of layers, the organic phase was dried (Na_2SO_4) and evaporated (N_2) to remove propanal and excess dimethylsulfide, followed by dilution by hexane. Part of this sample was used for subsequent field studies.

Field Trial with 9-Acetyloxynonanal. Synthetic 9-acetyloxynonanal was applied in six selected doses (0.3, 1, 3, 10, 100, or 1000 μg) to rubber septa in Peoria, Illinois, and sent to Montana, where they were stored at -80°C until use. Septa without added compound were the seventh (control) treatment. Beginning on June 23, 1999, a 15-day field test was conducted by one of us (D.K.W.) at a site 17 km northwest of Three Forks, Montana, near the county line between Jefferson and Broadwater counties (46°2.19′N, 111°38.39′W). The test was conducted as the sawfly population was beginning to decline. The field site consisted of a fence line that separated a standing crop of wheat (to the west) from a fallow field (to the east) that had been moderately infested with wheat stem sawflies during the 1998 growing season. This field was the source of the sawflies that would infest the growing crop. Prevailing winds were westerly; thus, movement into the new crop would generally be upwind. Twenty-one traps were spaced at 30-m intervals along this fence line. Each trap consisted of a 10-cm $\times$ 10-cm square of #20 mesh metal hardware cloth, coated with Tangle-Trap on both sides. The trap was stapled to a 60-cm wooden stake driven into the ground so that the bottom of the sticky surface was approximately 5 cm above the soil substrate. A rubber septum trap bait was penetrated with a paper clip and attached to the center of the upwind side of the sticky surface. Daily insect counts were taken and the trapped insects were removed. Lures were replaced and the traps were re-randomized every three days. Each of the seven dose treatments was replicated three times in the random trap array. The trap catch data were analyzed by Poisson regression, a method that is appropriate for small counts, using the Statistix analysis package (Analytical Software, 1998). Details of the models are given in the Results section.

RESULTS

Cuticular Extracts. GC-EAD analyses of male and female cuticular extracts were obtained with male and female antennae. As shown in Figure 1, GC-EAD analyses of female cuticular extracts and the simultaneously recorded EAD of male antennae showed no activity with any of the higher molecular weight compounds, such as the major cuticular hydrocarbons (Bartelt et al., 2002). However, compounds of lower molecular weight did stimulate male antennae. Similar results were obtained with female antennae and with male cuticular extracts and male and female antennae. Further research was focused on the analysis of volatiles collections due to the EAD activity of compounds of lower molecular weight.

Volatiles Collections from Individual Insects. Initial GC-EAD profiles of Super Q-collected volatiles from individual males and females revealed variable

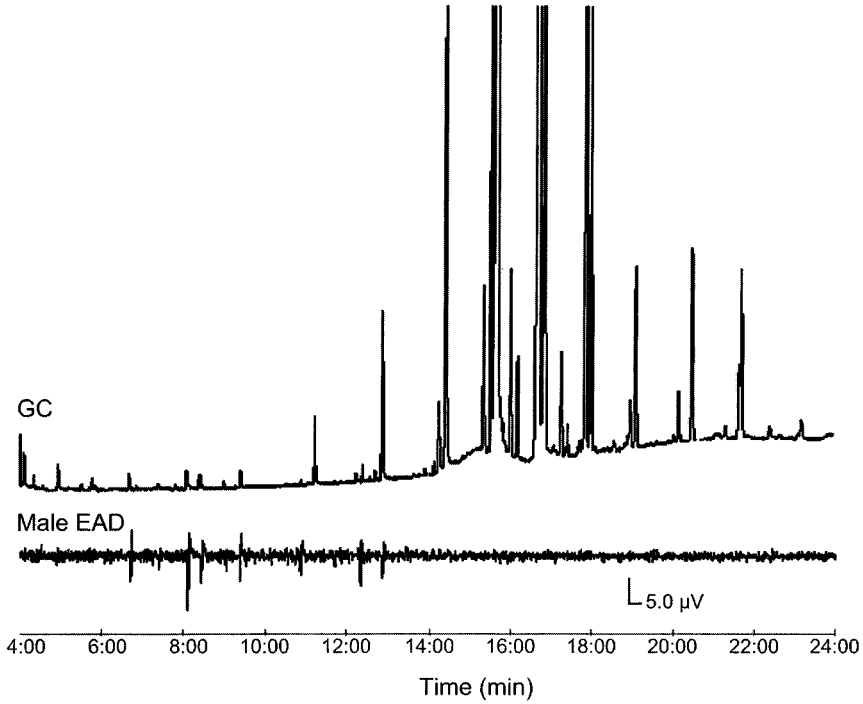


FIG. 1. Simultaneously recorded gas chromatogram (GC) and electroantennogram responses (EAD) of a typical male antenna to a cuticular hexane extract of female *C. cinctus*.

and relatively small amounts of insect-specific material during two- to three-day collection periods. In male-collected volatiles, the major compounds were nonanal and (*Z*)-9-tricosene (Bartelt et al., 2002). The same compounds, at lower amounts, were present in female-collected volatiles. With males, these compounds were present at 43 ng ($\pm$14 SD, $N = 8$) and 96 ng ($\pm$76 SD, $N = 8$) per sawfly per day, respectively. From females, collected amounts of nonanal were 26 ng ($\pm$13 SD, $N = 5$) per sawfly per day. Amounts of (*Z*)-9-tricosene from females were smaller (e.g., Figure 2), because females have less of this hydrocarbon than males (Bartelt et al., 2002). These initial male and female collections consistently elicited a notable depolarization on both male and female antennae (Figure 2). In addition to the depolarization elicited by the compound eluting at 10.42 min, several smaller depolarizations were noted. However, these depolarizations were less consistent, which was probably due to the variable and relatively small amounts of insect-specific material.

Identification of 9-Acetyloxynonanal. The mass spectrum of the compound giving the relatively strong depolarization (10.42 min) is shown in Figure 3. No

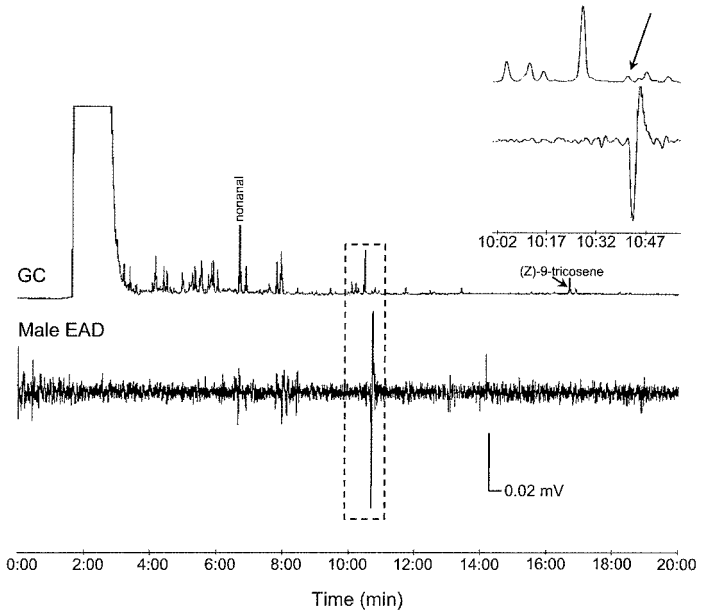


FIG. 2. Simultaneously recorded gas chromatogram (GC) and electroantennogram responses (EAD) of a typical male antenna to a volatiles collection from an individual female *C. cinctus*. Arrow in cutout denotes the location of 9-acetyloxynonanal.

molecular ion was visible, and the mass spectral library did not provide a probable identification. However, the fragment at *m*/*z* 61 was informative and suggested that the compound was a primary acetate. The largest significant ion in the chemical ionization (CI) mass spectrum had *m*/*z* 201, suggesting it was the protonated molecular ion; thus, the molecular weight was 200. The CI spectrum also had two major fragments. The first (*m*/*z* 141) represented a loss of 60 from the molecular ion and corresponded to a neutral loss of acetic acid, as would be anticipated for an acetate. The second fragment (*m*/*z* 123) represented an additional loss of 18, corresponding to a loss of water. Together, these features suggested the presence of three (or more) oxygen atoms for a possible molecular formula of $C_{11}H_{20}O_3$, with two sites of unsaturation. If two oxygens and one site of unsaturation were accounted for by an acetate function, then one additional oxygen and one double-bond equivalent would remain to be explained. Example spectra from the mass spectral library gave some guidance: in primary acetates with another functional group, the *m*/*z* 61 fragment peak is relatively large, as in the sawfly compound, only when the two functional groups are distant from each other in the structure. Thus, a nine-carbon chain with an acetate group on one end and an epoxy, aldehyde, or ketone group near the other end was suggested.

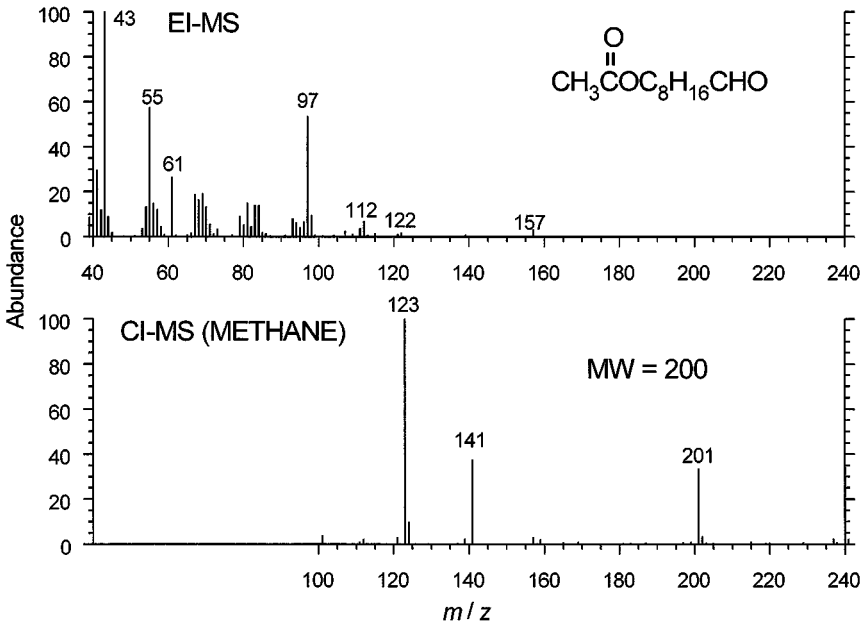


FIG. 3. Electron impact and chemical ionization mass spectra for 9-acetyloxynonanal.

Amounts of this compound collected from males and females were variable and were in the range of 1–5 ng per sawfly per day, with lower amounts produced by females overall. The amounts of material on hand were so small that other spectra or microchemical tests were not deemed feasible. The approach for continuing was to synthesize model compounds for comparison of mass spectral details and GC retention times. 9-Acetyloxynonanal was the first compound prepared because it was accessible in a single step (ozonolysis) from (*Z*)-9-dodecen-1-ol acetate, which was on hand as an example moth pheromone. Fortuitously, the synthetic and natural compounds matched perfectly, by GC retention time and mass spectrum. A larger amount of this compound was synthesized for subsequent laboratory and field use.

Volatiles Collections from Groups of Insects. Single males appeared odorless to the human nose and stayed relatively inactive when placed in collection tubes. However, placing several males together in a collection tube almost instantly triggered wing fanning of all the males, which was accompanied by the release of a relatively strong, distinctive odor. This odor was absent with single females and barely detectable with grouped females. Due to the detectable odor of grouped males, additional GC-EAD analyses were performed on volatiles from groups of insects, pooling collections to increase amounts of compound to be analyzed.

TABLE 1. WHEAT STEM SAWFLY (*Cephus cinctus*) VOLATILES IDENTIFIED FROM GROUPED SAWFLIES WITH ELECTROPHYSIOLOGICAL ACTIVITY ON MALE AND FEMALE ANTENNAE AND THEIR RELATIVE ABUNDANCE

Peak	Compound[a]	Relative abundance (mean ± SD)[b]	
		Per male/day	Per female/day
1	Nonanal[c]	100 ± 0	100 ± 0
2	Octanoic acid	29 ± 10	27 ± 6
3	Decanal	66 ± 9	63 ± 26
4	Phenylacetic acid	53 ± 43	3 ± 3
5	Nonanoic acid	53 ± 18	47 ± 12
6	Decanoic acid	35 ± 12	40 ± 31
7	Dodecanal	12 ± 1	10 ± 2
8	9-Acetyloxynonanal	10 ± 6	3 ± 2
9	Tridecanal	9 ± 2	7 ± 3
10	Tetradecanal	35 ± 20	14 ± 8
11	Pentadecanal	5 ± 1	9 ± 4
12	Hexadecanal	19 ± 13	32 ± 8
13	13-Acetyloxytridecanal	trace	trace

[a] Identification is based on comparison of mass spectra and retention times of the natural materials with those of authentic synthetic standards.
[b] Two- to three-day volatiles collections of groups of 5 males or females ($N = 5$).
[c] Male: 74 ng (±3 SD), Female: 37 ng (±7 SD).

GC analyses of volatiles from groups of females and the simultaneously recorded EAD activity of male and female antennae revealed 12 compounds (Table 1), that consistently stimulated the antennae (Figure 4). The same 12 compounds were present in volatiles collected from groups of males, and subsequent GC-EAD analyses confirmed their activity on male and female antennae (Figure 5).

The GC profiles differed notably between the sexes. Groups of males produced relatively more of the EAD-active compounds than groups of females on a per sawfly basis. The distinct odor released by grouped males was identified as phenylacetic acid, which was clearly more abundant with volatiles from males than from females. Ratios of aldehydes also differed. Male volatiles collections showed a relatively higher abundance of tetradecanal, whereas female collections showed higher amounts of hexadecanal. In addition. 9-acetyloxynonal was approximately three times more abundant in male collections than in female collections.

SPME-Collected Volatiles. The profile of SPME-collected volatiles from the first 30 min after placing five males together is shown in Figure 6. In males, the most abundant compounds were nonanal and tetradecanal. In females (not shown), hexadecanal replaced tetradecanal as the abundant larger aldehyde. GC-EAD profiles

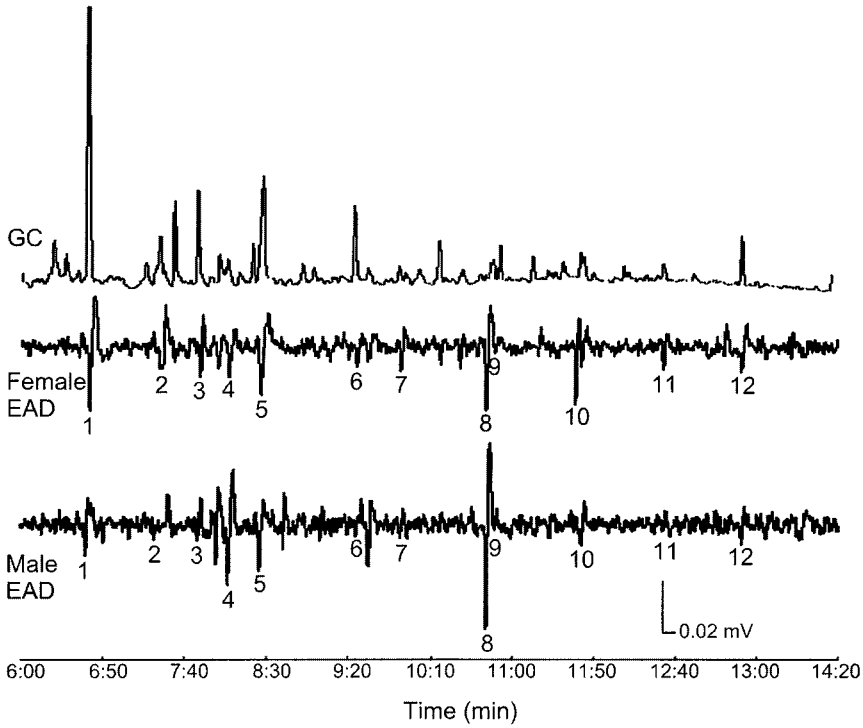


FIG. 4. Simultaneously recorded gas chromatogram (GC) and electroantennogram responses (EAD) of typical male and female antenna to volatiles collection on Super Q from a group of five female *C. cinctus*.

of SPME-collected volatiles confirmed the EAD-activity of the 12 compounds collected with Super Q.

Ozonolysis of Cuticular Extracts. The extract shown in Figure 1 showed relatively small amounts of EAD-active materials. However, this same extract showed a dramatic increase in EAD-active compounds after ozonolysis. The presence of these EAD-active compounds was confirmed by GC-MS and GC-EAD analysis (Figure 7). The most abundant compound after ozonolysis was nonanoic acid. 13-Acetyloxytridecanal, which was present in trace amounts in some of the volatiles collections and was without detectable antennal activity, consistently stimulated male and female antennae in ozonized extracts (peak 13, Figure 7).

Field Trial with 9-Acetyloxynonanal. The trap catch totals and means for the various doses of 9-acetyloxynonanal are shown in Table 2. The sex ratio was nearly 1:1, with 179 females and 164 males being captured. No pattern could be discerned between sex ratio and trap treatment; thus, the data were pooled over sex. The

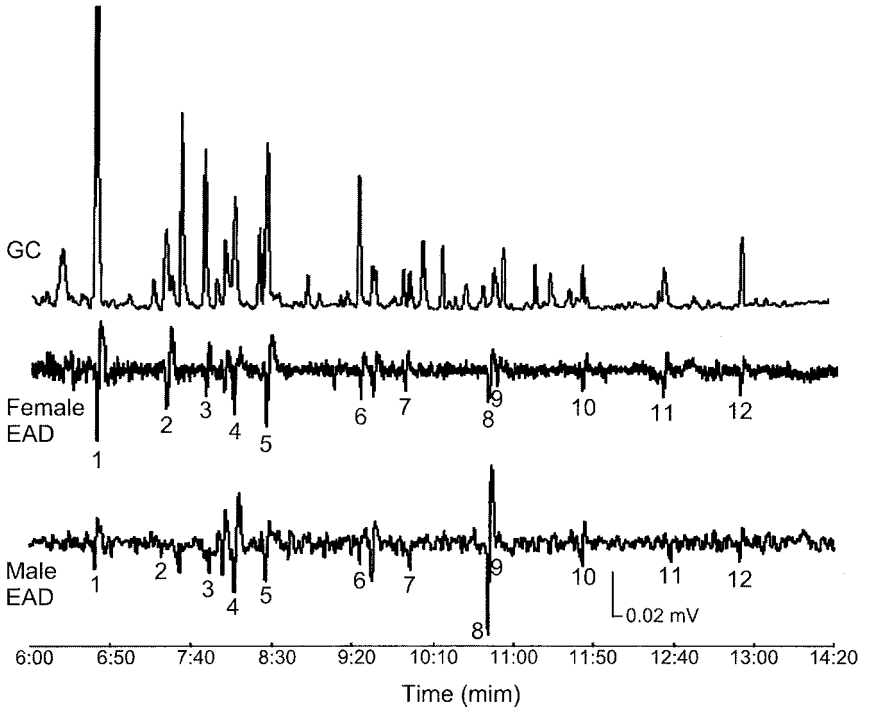


FIG. 5. Simultaneously recorded gas chromatogram (GC) and electroantennogram responses (EAD) of typical male and female antenna to volatiles collection on Super Q from a group of five male *C. cinctus*.

initial Poisson regression model applied to the trap catch data (315 observations) included terms for differences among days of the experiment (14 *df*), differences among treatments (6 *df*), and the constant term (1 *df*). The resulting likelihood ratio statistic (G^2) for the model was 409.6 (294 *df*, $P < 0.0001$, compared to the χ^2 distribution). Thus the overall residual variance was larger than expected for a Poisson process. Examination of residuals revealed that two of the catches for the 1000-μg treatment were inconsistently large: 59 sawflies in one trap on day 2 and 13 sawflies in one trap on day 5. When these two data points were removed as outliers, the model fitted adequately ($G^2 = 303.9$, 292 *df*, $P = 0.30$), and conditional tests (Fienberg, 1977) for differences among treatments or days were then justified. The overall test for differences among treatments was highly significant ($G^2 = 147.2$, 6 *df*, $P < 0.00001$). Differences among days were also highly significant ($G^2 = 146.4$, 14 *df*, $P < 0.00001$). Averaged over all treatments and replications, mean daily trap catches for the 15 days of the study ranged from

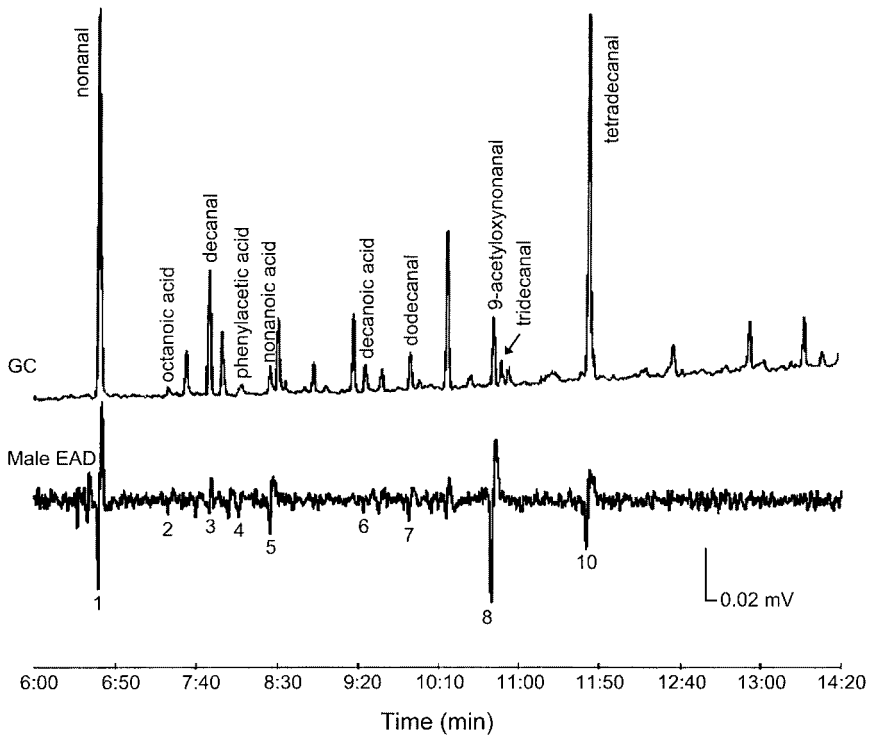


FIG. 6. Simultaneously recorded gas chromatogram (GC) and electroantennogram responses (EAD) of a typical male antenna to volatiles collected with solid phase microextraction (SPME) of the first 30 min when five male *C. cinctus* were grouped together.

0.11 to 1.23; it was necessary to allow for differences among days in the model. Daily sawfly activity is greatly influenced by weather conditions (temperature, wind, and cloud cover; D. K. Weaver, personal observation).

Differences among treatments were further explored. A linear relationship was fitted for log catch versus log dose; a separate term (1 *df*) was simultaneously fitted for the control because the logarithm for dose = 0 would be meaningless. The linear trend was highly significant ($G^2 = 107.4$, 1 *df*, $P \ll 0.00001$), and the value of the slope corresponded to a 1.70-fold increase in trap catch for each 10-fold increase in septum dose. Deviations from the linear trend were also significant ($G^2 = 11.45$, 4 *df*, $P = 0.022$), but these were relatively minor and did not suggest that any simple curve would describe the data better than the straight line. A series of 1-*df* comparisons were made between individual doses and the control to define the response threshold. As shown in Table 2, all doses

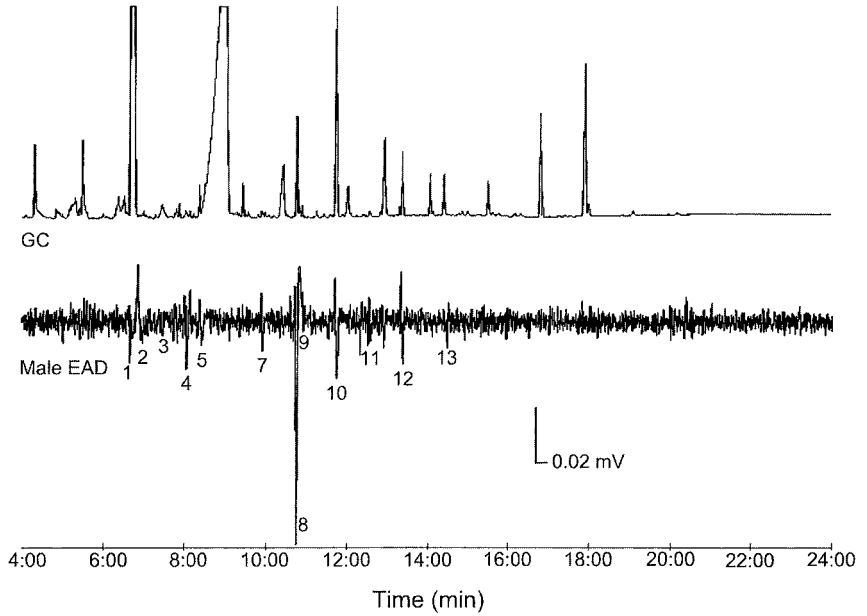


FIG. 7. Simultaneously recorded gas chromatogram (GC) and electroantennogram responses (EAD) of a typical male antenna to a cuticular hexane extract of female *C. cinctus* after ozonolysis.

of 9-acetyloxynonanal ≥ 3 μg/septum were significantly more attractive than the control. This data set demonstrated a consistent, although fairly subtle, field activity of 9-acetyloxynonanal. Although the highest dose, 1000 μg, was clearly the most active, activity became detectable at 3 μg.

TABLE 2. SUMMARY OF FIELD TRAPPING EXPERIMENT WITH 9-ACETYLOXYNONANAL

Septum dose (μg)	Total catch (N)	Mean catch	G^2 for difference from control (P value, 1 df)
0 (control)	12 (45)	0.27	
0.3	20 (45)	0.44	2.02 (0.16)
1	15 (45)	0.33	0.33 (0.57)
3	33 (45)	0.73	10.19 (0.0014)
10	39 (45)	0.87	15.05 (0.0001)
100	43 (45)	0.95	18.54 (0.00002)
1000	106 (43)[a]	2.47	94.18 ($\ll$0.00001)

[a] Two outlier data points were removed, which had values of 59 and 13 (see text).

The conclusion from the analysis was that the effect of 9-acetyloxynonanal was real, even after two of the highest trap catches had been removed as outliers. An alternative analysis that would legitimately make use of all trap data (but that would probably be more complex than Poisson regression) would be expected to demonstrate the effect of the compound even more strongly.

DISCUSSION

Both males and females release the same EAD-active compounds. Although there was no evidence for any sex-specific compounds, overall release of EAD-active compounds was higher with grouped males than with grouped females. This was especially noted for phenylacetic acid, which was instantly detectable by the human nose when male sawflies were put together. In addition, male and female volatiles collections differed in the ratios of 9-acetyloxynonanal, tetradecanal, and hexadecanal. In the absence of qualitative differences, these different male and female ratios might be important clues for sex recognition.

The EAD-active 9-acetyloxynonanal is part of the pheromone of *C. cintus*. This compound alone attracted both male and female sawflies in the field bioassay. However, as is evident from our results, the complete pheromonal system of *C. cintus* appears to be more complicated. Our volatiles collections showed that males and females release 13 EAD-active compounds and, with the exception of phenylacetic acid, all could be derived through oxidation of cuticular compounds (Bartelt et al., 2002). Higher molecular weight compounds in male and female cuticular extracts showed no GC-EAD activity and the relatively small amounts of compounds of lower molecular weight showed relatively weak EAD responses. However, extracts after ozonolysis showed dramatically increased amounts of aldehydic GC-EAD-active compounds and absence of the unsaturated, higher-molecular-weight materials, giving credibility to the notion of an oxidative origin of the GC-EAD active compounds. In particular, unsaturated cuticular compounds could function as the precursors for the EAD-active compounds by oxidative cleavage of their double bonds (Bartelt et al., 2002). One might expect the oxidation of cuticular monoolefins to be a relatively slow process. However, EAD-active materials could be detected within minutes rather than hours using SPME, suggesting that an oxidative system might be fast enough to be significant to the sawflies' sexual communications under field conditions.

A multitude of factors can trigger and influence the abiotic oxidation of unsaturated cuticular compounds, making this a complicated process to study (Bartelt et al., 2002). For example, the action of ultraviolet light and the effect of temperature, as well as premating behaviors such as wing fanning and rubbing of the abdomen (D. K. Weaver, personal observations) could all influence this oxidation and will need further attention. Preliminary experiments showed that

abiotic oxidation of alkenediol diacetates, present on the cuticle of the insects, could yield the biologically active acetyloxyaldehydes (Bartelt et al., 2002). However, enzymatic involvement in *C. cinctus* pheromone system has not been ruled out, especially with regard to the occurrence of phenylacetic acid.

All the pheromones of sawfly species have thus far been reported as being female-produced. For example, sex attraction in the genera *Diprion* and *Neodiprion* depends to a large extent on esters derived from various optical isomers of female-produced 3,7-dimethyl-2-pentadecanol and from homologous alcohols with carbon chains of different length (Anderbant, 1999, and references therein). A different pheromone system has been reported for the tenthredinid, *Pikonema alaskensis* (Rohwer), still female-produced, but derived from the oxidation of sex-specific straight-chain hydrocarbon dienes found on the cuticle of the insects (Bartelt and Jones, 1983; Bartelt et. al., 1983). Although restricted to one sex, the oxidative component in the pheromone system of *P. alaskensis* resembles that of *C. cinctus*.

Field observations have shown that males form in-flight aggregations and that female sawflies near these active aggregations are rapidly recognized by male *C. cinctus*, and synthetic 9-acetyloxynonanal released from rubber septa can induce this aggregation (D. K. Weaver, unpublished). Furthermore, from the electrophysiological data, it is likely that more dramatic responses would result if additional antennally active compounds were included in the trap baits. Finally, trap design was a critical issue in this study. A number of trap types were tried (D. K. Weaver, unpublished) but were unsuccessful either because the insects would not enter a trap with interior shadows or because the trapping surface was so large and accessible that frequent accidental captures overwhelmed the treatment effects. While the trap design employed here was suitable for the present study, future improvements would be desirable.

9-Acetyloxynonanal has not been reported as an insect attractant before, but this and other acetyloxyaldehydes were analytically useful as cleavage products of epoxides in a method to locate the double-bond position in long-chain acetates (Bierl-Leonhardt et al., 1980). The mass spectrum of 9-acetyloxynonanal reported by Bierl-Leonhardt et al. (1980) did not agree with ours. However, this discrepancy might be due to a typographical error in the above-mentioned paper, as the published spectrum was also inconsistent with the spectra of homologous aldehyde acetates in that report.

The behavioral role of phenylacetic acid has yet to be determined. Its presence was noted in volatiles collections from both sexes, and its odor was instantly detected by the human nose when males were grouped together. Phenylacetic acid has not been reported as an insect attractant. Laboratory bioassays have shown that female *C. cinctus* were attracted to volatiles emitted from groups of males, but not to volatiles from single males (D. K. Weaver, unpublished). Whether this attraction to groups of males is due to the release of phenylacetic acid or due to a threshold effect is not clear and will need further study.

Individually, none of the identified EAD-active compounds would account for recognition of females by males. Therefore, future research will focus on more behaviorally driven assays of collections in order to collect a possible distinct female-specific chemical signal. In addition, emphasis will be given to elucidate the behavioral significance of additional EAD-active compounds and the importance of different ratios of EAD-active volatiles between the sexes.

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SEX PHEROMONE OF SOUTH AMERICAN TORTRICID MOTH *Argyrotaenia sphaleropa*

S. NUNEZ,[1] J. J. DE VLIEGER,[2,]* J. J. RODRIQUEZ,[1] C. J. PERSOONS,[2] and I. SCATONI[3]

[1]*INIA*
Las Brujas, P.O. Box 33085
Las Piedras, Canalones, Uruguay

[2]*TNO Industrial Technology*
P.O. Box 6235
5600 HE, Eindhoven, The Netherlands

[3]*Facultad de Agronomía, Cátedra de Entomología*
Garzon 780
12900 Montevideo, Uruguay

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Abstract—By means of electroantennographic detection and gas chromatography-mass spectrometry, the sex pheromone of *Argyrotaenia sphaleropa* was identified as a mixture of (*Z*)-11-tetradecenal, (*Z*)-11,13-tetradecadienal, (*Z*)-11-tetradecenyl acetate, and (*Z*)-11,13-tetradecadienyl acetate in the ratio of 1:4:10:40. Best trap catches were obtained with mixtures of (*Z*)-11-tetradecenal and (*Z*)-11,13-tetradecadienal in the ratio of 1:4 to 1:9.

Key Words—Pheromones, *Argyrotaenia sphaleropa*, (Z)-11-tetradecenal, (Z)-11,13-tetradecadienal.

INTRODUCTION

The native leafroller *Argyrotaenia sphaleropa* (Meyrick) is an important pest of deciduous fruit crops and grapes in Uruguay. The species occurs in the south of Brazil, Argentina, Bolivia, and Peru. (Bentancourt and Scatoni, 1995). Three to four insecticide sprays per season are necessary to control this pest, but in the case of conventional orchards, the organophosphate insecticide sprays (5-8 per season) aimed at controlling the codling moth (*Cydia pomonella*) also control this leafroller.

*To whom correspondence should be addressed.

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However, in orchards where mating disruption of the codling moth is part of the integrated pest management system, this leafroller has become a severe pest. Under these conditions, monitoring is the key tool for rational control. Malt-baited traps have been used, but operating these traps under practical conditions is difficult, time consuming, and not always reliable. Therefore, it was decided to identify the pheromone of this leafroller and to develop a monitoring system based on pheromones. A first attempt to identify the pheromone of *A. sphaleropa* was undertaken by the Japanese International Cooperation Agency (JICA) (Nunez, unpublished data). The work of this group was seriously hampered by the fact that no living insects were allowed to be imported into Japan. Biomonitoring of purification procedures [electroantennography (EAG) gas chromatography–electroantennographic detection (GC-EAD)] and/or wind-tunnel experiments, therefore, could not be performed. Based on GC-MS only, they tentatively identified three compounds: tetradecyl acetate (14:Ac), (*Z*)-11-tetradecenyl acetate (*Z*11–14:Ac), and (*Z*,*E*)-9-11-tetradecadienyl acetate (*Z*9,*E*11–14:Ac). However, when field-trapping experiments were carried out with the above-mentioned compounds in various ratios and quantities, no male moths were trapped. Hence, it was concluded that the identification either was incomplete or that the proposed structure(s) for one (or more) of the compounds identified was (were) incorrect.

Within the framework of the European Commission (Cooperation with nonmember countries, Project CI*-CT94-0006), a joint project TNO-INIA was undertaken to characterize the sex pheromone of *A. sphaleropa*.

METHODS AND MATERIALS

Rearing of Insects. A. sphaleropa was reared on a synthetic diet (Shorey and Hale, 1965) under reversed long-day conditions. (14L:10D) at 24°C and 60% relative humidity. Two cultures both at INIA and at TNO were maintained simultaneously. Adults were maintained on a mixture of water and honey. To avoid degeneration of the culture, fresh material was collected regularly in the field and introduced into the laboratory culture.

Pheromone Extraction. Field experiments with female-baited traps had indicated that most *A. sphaleropa* moths were trapped shortly after midnight and, therefore, this period was considered to be the period of maximum pheromone production. Pheromone glands were clipped during or shortly after this period. Females 2–3 days old were anesthesized with carbon dioxide, and by gently pressing the abdomen, the pheromone gland was extruded and could be clipped without any appendages. The dissected glands were collected in hexane (10–20 μl/gland) and shaken regularly for 10–15 min. The hexane extract was kept at -20°C.

Electroantennography (EAG). Based on the findings of the Japanese workers, *A. sphaleropa* was subjected to EAG, using a series of 21 monounsaturated and

8 double unsaturated tetradecenyl acetates (10-μg samples on filter paper pads); samples were freshly prepared; the purity of the compounds was >97% by GC analysis. The experiment was repeated with 1-μg samples per filter paper pad.

Gas Chromatography–Electroantennographic Detection (GC-EAD). A gas chromatograph (GC) was fitted with capillary columns at the end of which an effluent splitter (1:15) was installed. To achieve proper elution of the compounds, 30 ml N_2 /min was introduced between the column and the splitter (makeup gas). Five percent of the compounds eluting from the column were fed to the FID, whereas 95% of the compounds were transferred by means of capillary tubing through the heated outlet of the GC into a mixing chamber. The outlet of the mixing chamber (glass tubing; 1 cm diam.) was directed towards the antennae. By blowing air into the mixing chamber, the compounds eluting from the column were blown across the antennae (Venturi effect). The linear air velocity at the antennae was 0.6 m/sec. The signals derived from the antennae and from the flame ionization detector (FID) were simultaneously fed to a computer linked to a display and a printer. The traces on the display of the two signals enabled us to correlate directly the EAD signals with the FID signals (Syntech, Hilversum, The Netherlands). Amounts of *ca*. 4 female equivalents (FE) were injected on to a DB-23 column (25 m $\times$ 0.53 mm ID; He carrier gas at 6 ml/min; makeup gas 30 ml/min; split ratio 1:15; oven temperature 170°C; injection port 200°C; detector 225°C; outlet 200°C). The same experiments were repeated on a CPSil-5 column (25m $\times$ 0.53 mm ID; temperature programmed from 120°C to 220°C at 10°C/min; 5 min isotherm at 220°C) and on a CPWax-52 column (temperature programmed for 7 min at 165°C and increased to 200°C at 10°C/min; 5 min isotherm at 200°C); the other conditions were as mentioned above.

Gas Chromatography–Mass Spectrometry (GC-MS). A VG-250S mass spectrometer fitted with either a CPWax-52 or CPSil-5 column was used (50 m $\times$ 0.32 mm ID; $df = 1$ μm). MS spectra were run on a CPSil-5 column (temperature programmed from 80°C to 250°C at a rate of 15°C/min or on a CPWax-52 column (190°C). Both EI and CI mass spectra were recorded. For CI spectra, methane was used and the ion source set at 200°C. In a later stage, the ion source temperature was set at 100°C.

Field Evaluations. Wing-type traps with replaceable sticky bottoms were loaded with different amounts and different ratios of the pheromone blends on polyethylene or rubber dispensers. Each treatment had two to five replicates, and traps were randomly installed. The distance between traps was at least 25 m to avoid interferences. Traps were rotated every week, and the trapped insects were counted. Virgin female traps were used as a control and were baited with two virgin females. These females were replaced twice a week.

For statistical analysis, captures were grouped per period of evaluation, transformed to square root, and analyzed by ANOVA followed by Duncan test ($\alpha = 0.05$).

RESULTS

The EAG experiments with the series of mono- and double-unsaturated tetradecenyl acetates indicated that (*Z*)-11-tetradecenyl acetate (*Z*11–14:Ac) evoked the most intensive EAG amplitude. EAG amplitudes evoked by the other two compounds, tentatively identified by the Japanese group (Nunez, unpublished data) as tetradecanyl acetate (14:Ac) and (*Z*,*E*)-9,11-tetradecadienyl acetate (*Z*9,*E*11–14:Ac) were far less intensive (10 μg on filter paper pads). When 1-μg samples were tested, *Z*11–14:Ac still evoked the highest EAG amplitude, whereas at this concentration, 14:Ac and *Z*9,*E*11–14:Ac barely gave EAG responses. This suggested that *Z*11–14:Ac might be part of the pheromone, whereas the findings with regard to 14:Ac and *Z*9,*E*11–14:Ac suggest that these compounds are not part of the pheromone complex.

Injection of 4 (FE) of *A. sphaleropa* on the DB-23 column gave four reproducible AD responses with retention times (R_ts) of 2.05, 2.38, 2.53, and 3.40 min, respectively. The EAD active fraction eluting at 2.05 min from the DB-23 column was collected in a chilled capillary and reinjected on a CPWax-52 column (Burson and Kenner, 1969). On the latter column, its R_t was 5.14 min. Under the same conditions, *E*11–14:Ald and *Z*11–14:Ald eluted at 5.03 and 5.16 min, respectively. Comparison of the MS of the natural compound with the MS of synthetic *Z*11–14:Ald, recorded on a CPWax-52 column, confirmed the natural compound to be a monounsaturated 14:Ald ($M = 220$). Based on these data, it was concluded that this compound was *Z*11–14:Ald.

The R_t of the EAD response at 2.38 min on the DB-23 column corresponded with the R_t of *Z*11–14:Ac. Both its EI and CI mass spectra (CPSil-5 column) confirmed the compound to be a monounsaturated 14:Ac ($M = 254$). Both mass spectra of the natural compound were in full agreement with the mass spectra of synthetic *Z*11–14:Ac.

The compound eluting from the DB-23 at 2.53 min was collected in a chilled capillary and re injected on a CPSil-5 column (Burson and Kenner, 1969). On this column, its R_t was 2.23 min. The EI spectrum (on CPSil-5) showed a strong fragment at m/z 54. This fragment is typical for conjugated diene systems with a terminal double bond (Ando et al., 1985, 1988). Its CI mass spectrum showed the molecular weight to be 208. Combining these data leads to the conclusion that this compound might be an 11,13–14:Ald. The EI spectrum of synthetic *Z*11,13–14:Ald (kindly provided by Dr. T. Ando, Tokyo University of Agriculture and Technology, Tokyo, Japan and Dr. A. Cork, NRI, Chatham, UK.) gave a 100% match with the EI spectrum of the natural compound. As the amounts of some of the compounds in the natural extract were extremely low, determination of the configuration of 11,13–14:Ald based on R_t was impossible. We, therefore, used the GC-EAD technique. Injection of a very low dose of *E*- and *Z*11,13–14:Ald on CPSil-5 and DB-23 indicated that the EAD response to the *Z* isomer was

consistently much higher than to the *E* isomer. Based on these data, it was concluded that the 11,13–14:Ald had the *Z* configuration. The compound eluting from the DB-23 column at 3.40 min was collected in a chilled capillary and reinjected on a CPSil-5 column (Burson and Kenner, 1969). On the latter column, its R_t was 4.15 min. Its EI and CI spectra (on CPSil-5) suggested this compound to be a double unsaturated 14:Ac [$M = 252$; M-60 (acetate) $=192$]. A major fragment, m/z 54 (see also *Z*11,13–14:Ald), indicated that the compound might be a conjugated diene system with a terminal double bond and could be either *Z*- or *E*11,13–14:Ac. Comparison of the EI spectrum of synthetic *Z*11,13–14:Ac (kindly provided by Dr. J. Klun, USDA, Beltsville, Maryland) with the spectrum of the natural compound confirmed this hypothesis.

Determination of the configuration was performed by GC-EAD. The same procedure was followed as was used for the determination of the configuration of 11,13–14:Ald. However, the responses to *Z*11,13–14:Ac and *E*11,13–14:Ac were low. Based on biosynthetic considerations, it was assumed that the 11,13–14:Ac had the same configuration as the 11,13–14:Ald, i.e., the *Z* configuration. This leads to the same conclusion that the pheromone of *A. sphaleropa* consists of a mixture of *Z*11–14:Ald, *Z*11,13–14:Ald, *Z*11–14:Ac, and *Z*11,13–14:Ac (ratio of 1:4:10:40). GC-EAD of a synthetic mixture as mentioned above on both a CPSil-5 and a DB-23 column gave a complete match with the natural extract.

Field Evaluation of Different Pheromone Blends. The results of a first field experiment are summarized in Table 1. Dispensers loaded with 1 mg of a blend of *Z*11–14:Ald and *Z*11,13–14:Ald in a ratio of 1:4 show the highest attraction, comparable to traps baited with two females. Addition of *Z*11–14:Ac and of *Z*11,13–14:Ac to this blend seems to inhibit attraction. This might be due to the fact that *Z*11–14:Ac inhibits attraction or due to the lower concentration

TABLE 1. *Argyrotaenia sphaleropa* MOTHS CAPTURED PER TRAP[a]

Pheromone blend tested				Dose (mg)	Average catch/ trap
*Z*11–14:Ald	*Z*11,13–14:Ald	*Z*11,13–14:Ac	*Z*11–14:Ac		
1	4	20	10	2	10.6d
1	4	20	10	1	6.2d
1	4	20	10	0.5	2.6d
1	4			1	115.0ab
	1		3	1	22.6cd
1	4		10	1	49.0bc
Virgin female traps (2 per trap)					125.0a

[a] Average number caught between January 20 and February 15, 1996. Traps loaded with different pheromone blends in polyethylene dispensers, three replicates. Means followed by the same letter are not significantly different ($P > 0.05$, Duncan's test).

of the diene when *Z*11–14:Ac is added: 1 mg of the blend of *Z*11–14:Ald and *Z*11,13–14:Ald (ratio 1:4) contains 0.8 mg of the diene, whereas 1 mg of the blend *Z*11–14:Ald, *Z*11,13–14:Ald, and *Z*11–14:Ac (1:4:10) contains 0.27 mg of the diene. To determine more precisely the role and the most attractive ratio of the two aldehydes, a new series of blends was tested, using polyethylene and rubber dispensers (Table 2). The results in Table 2 show that *Z*11,13–14:Ald is attractive alone. However, its attractiveness is increased by adding *Z*11–14:Ald. The best ratio *Z*11–14:Ald, *Z*11,13–14:Ald seems to be 1:9. This ratio is even slightly more atractive than two virgin females. For the same pheromone rate and ratio, the differences of moths captured with rubber or polyethylene dispensers are not significant. As this experiment was carried out at the end of the flight period of *A. sphaleropa*, catches are lower than those reported in Table 1.

DISCUSSION

Of the compounds tentatively identified by the Japanese workers as part of the pheromone of *A. sphaleropa*, only the presence of *Z*11–14:Ac could be confirmed. The presence of tetradecyl acetate and of (*Z*,*E*)-9,11-tetradecadienyl acetate (*Z*9,*E*11–14:Ac) could not be confirmed. Comparison of the mass spectrum provided by the Japanese workers (Nunez, unpublished data), to which they tentatively assigned *Z*9,*E*11–14:Ac as the structure, with that of synthetic

TABLE 2. AVERAGE NUMBER OF *Argyrotaenia sphaleropa* CAPTURED PER TRAP BETWEEN MARCH 15 AND MAY 15, 1996[a]

Pheromone blend tested		Type of dispenser[a]	Traps (*N*)	Dose (mg)	Average catch/trap
Z11-14:Ald	Z11,13-14:Ald				
100	—	P	3	1	0.6 d
100	—	R	2	1	0.0 d
—	100	P	3	1	30.0 ab
—	100	R	2	1	13.0 bc
50	50	P	5	1	2.6 d
50	50	R	5	1	3.6 cd
20	80	P	5	1	21.2 b
20	80	R	5	1	31.2 ab
10	90	P	5	1	64.0 a
10	90	R	4	1	51.0 a
Virgin female traps (2 per trap)			2		48.5 ab

[a]R, rubber dispenser; P, polyethylene dispenser. Means followed by the same letter are not significantly different ($P > 0.05$, Duncan's test).

*Z*9,*E*11–14:Ac (from our own stock) showed some marked differences. The mass spectrum of the compound isolated by the Japanese workers showed a strong *m*/*z* 54 fragment, whereas the mass spectrum of *Z*9,*E*11–14:Ac did not. This fragment is typical for conjugated diene systems with a terminal double bond (Ando, et al., 1985,1988). The mass spectrum provided by the Japanese workers to which they assigned *Z*9,*E*11–14:Ac as the structure looked similar to the mass spectrum of *Z*11,13–14:Ald. Therefore, it might well be that the Japanese workers were dealing with the correct compound, but that an incorrect structure was assigned to it. *Z*11,13–14:Ald and *Z*11,13–14:Ac have not been found before as part of a pheromone blend, whereas the *E* isomers of these compounds have been found as part of the pheromone of *Acleris minuta* (Schwarz et al., 1983). Moreover, in field screening tests, *E*11,13–14:Ald has been shown to be attractive for a number of *Acleris* sp. (Ando et al., 1987).

Although it was found that the major compound (*Z*11,13–14:Ald) was attractive alone, trap catches increased with mixtures of two pheromone compounds relative to either component alone. Binary mixtures as sex pheromones are well known, and this has repeatedly been established for Tortricidae (Roelofs and Cardé, 1974; Persoons and Ritter, 1975; Ritter and Persoons, 1976), where it seems to be more the rule than the exception. However, binary mixtures are also found in other moth families (Mayer and McLaughlin, 1991). In those cases where synergistic effects of binary pheromone mixtures have been observed, the individual compounds are barely and often not attractive at all in field experiments.

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FIELD VERIFICATION OF PREDATOR ATTRACTION TO MINNOW ALARM SUBSTANCE

BRIAN D. WISENDEN* and TRAVIS A. THIEL

Biology Department
Minnesota State University, Moorhead
Moorhead, Minnesota 56563

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Abstract—Fishes such as minnows in the superorder Ostariophysi possess specialized alarm substance cells (ASC) that contain an alarm cue. Alarm substance can only be released by damage to the epidermis; thus, the release of alarm substance is a reliable indicator of predation risk. When nearby minnows detect the cue, they adopt a range of antipredator behaviors that reduce their probability of predation. Predator–predator interactions afford prey an opportunity to escape and, thus, a fitness benefit that maintains alarm substance calls over evolutionary time. Here, we present data from a simple field experiment verifying that nearby predators are attracted to minnow alarm substance because it signals an opportunity to pirate a meal. Fishing lures were baited with sponge blocks scented with either (1) water (control for sponge odor and appearance), (2) skin extract from non-ostariophysan convict cichlids (superorder Acanthopterygii, *Archocentrus "Cichlasoma" nigrofasciatus*) to control for general injury-released cues from fish, or (3) skin extract from fathead minnows (superorder Ostariophysi, *Pimephales promelas*). Predator strike frequency on each sponge type was 1,1, and 7 for water, cichlid, and minnow cues, respectively. These data provide the first field test using fish predators of the predator-attraction hypothesis for the evolution of Ostariophysan alarm substance cells.

Key Words—*Schreckstoff*, alarm substance, alarm pheromone, minnow, predator attraction, evolution, chemical signal, predator–prey, Ostariophysi.

INTRODUCTION

Predation, and risk of predation, influences the evolution of behavior and morphology of virtually all animals because predation is the final arbiter of natural

*To whom correspondence should be addressed. E-mail: wisenden@mnstate.edu

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selection (Lima and Dill, 1990). Chemical cues mediate predator–prey interactions in aquatic habitats for organisms ranging from ciliates to mammals (Wisenden, 2001). Chemical cues released by predators, or from conspecifics that have been injured or frightened, are used by prey to detect and evade predators (Smith, 1992, 1999; Kats and Dill, 1998; Chivers and Smith, 1998; Wisenden, 2000).

Predators cause physical damage and injury to their prey in the process of capture, handling, and ingestion. Consequently, chemical cues from injured prey are often released during a predator attack, and these cues serve as public information about predation risk. Most aquatic taxa, from ciliates to amphibians, exhibit antipredator behavior in response to injury-released chemical cues from conspecifics (Chivers and Smith, 1998).

Fishes in the superorder Ostariophysi, i.e., minnows, catfish, characins, and suckers, represent about 64% of all freshwater fish species (Nelson, 1994). One character that unites this group is the presence of specialized epidermal cells that contain an alarm substance; hence, the cells are referred to as alarm substance cells (ASCs). These cells lack an external duct. The cell contents are released only when the skin is damaged. Predator attacks on Ostariophysans release alarm substance into the surrounding water and warn nearby prey of predation risk. Alarm substance is potent. Minnow skin elicits overt antipredator behavior when diluted to 1 cm^2 skin per 58,000 liters (Lawrence and Smith, 1989), or the equivalent volume of a sphere with a radius of 3.7 m. The behavioral response to this cue significantly reduces the probability of predation risk (Mathis and Smith, 1993).

ASCs have caught the attention of evolutionary ecologists. It is not readily apparent how the alarm signaling function of ASCs provides a selfish benefit to the signal sender, and thereby provides a mechanism for these cells to be maintained by natural selection (Smith, 1992; Williams, 1992). The only evolutionary mechanism for ASCs for which there is experimental support is the predator-attraction hypothesis (Smith, 1992). In a laboratory experiment, northern pike *Esox lucius* were more attracted to ASC–minnow skin extract than skin extract without ASCs (Mathis et al., 1995). Similarly, in a field experiment, dytiscid predaceous diving beetles were more attracted to traps scented with minnow skin with ASCs than traps scented with minnow skin that lacked ASCs (Mathis et al., 1995). Chivers et al. (1996) showed in staged predation events in laboratory aquaria that fathead minnows in the grasp of a small pike have a significant chance of escape if a second, slightly larger, pike is allowed to interfere. Fathead minnows receive a selfish fitness benefit by investing in ASCs because ASCs attract secondary predators, not because they warn conspecifics of predation risk. The studies by Mathis et al. (1995) and Chivers et al. (1996) make important contributions to the understanding of the evolution of chemical alarm signaling in this ecologically important group of fishes. No other experiments

have been conducted to verify these findings, and importantly, they have not been verified with fish predators in the field. In this study, we report the results of a simple field experiment that provides further support for the secondary predator-attraction hypothesis.

METHODS AND MATERIALS

We conducted our experiment on Lake Ida, approximately 25 km north of Alexandria, Minnesota, USA (46°00′ N, 95°25′ W). Potential minnow predators in Lake Ida are: northern pike, *Esox lucius*; walleye, *Stizostedion vitreum vitreum*; largemouth bass, *Micropterus salmoides*; yellow perch, *Perca flavescens*; black crappie, *Poxomis nigromaculatus*; several types of bullhead catfish, *Ameiurus melas, A. nebulosus, A. natalis*; and perhaps bluegill sunfish, *Lepomis machrochirus*.

We fished for 4 hr on January 27 and 4 hr on February 11, 2001. Ice cover at the time of data collection was about 80 cm thick. We drilled six holes about 25 cm in diameter with a gas-powered auger and spaced the holes about 8 m apart. We used a depth sonar to standardize water depth below all holes at about 3 m. We fitted each hole with an ice-fishing tip-up. A tip-up has a small platform and an upright stand connected to an arm that extends over the hole. The arm was equipped with a reel of anti-freezing line followed by a 1.1 m length of transparent monofilament line "leader." A lure was attached to the end of the leader. At the other end of the arm there was a piece of springy metal attached to a fluorescent orange flag. The flag was flexed against the reel of line in such a way that when a fish tugs on the line the flag is released, signaling a strike. In addition, a broad metal paddle was attached to the arm designed to catch any wind movement, causing the arm to bob up and down and jig the line. Jerky vertical motion on the line attracts fish to the lure. All holes were about 300 m from shore and equally exposed to wind. By using wind-powered tip-ups, we standardized the visual presentation of test stimuli among treatments.

Each tip-up was fitted with a fluorescent green, 1/32 oz (0.9 g) teardrop style jig and a small block of cellulose sponge ($1 \times 1 \times 0.7$ cm). The sponge block was soaked with one of three test solutions: (1) dechlorinated tap water to control for the odor and appearance of the sponge and background attractiveness of the jig lure, (2) skin extract of a tropical fish (convict cichlid, *Archocentrus "Cichlasoma" nigrofasciatus*) to control for general chemical cues released from injured fish, or (3) skin extract of fathead minnows, *Pimephales promelas*. Convict cichlids are not in the Ostariophysi (superorder Acanthopterygii) and, therefore, do not possess Ostariophysan ASCs. Skin extracts for each species were prepared using the same protocol. We used five cichlids (mean $\pm$ SE $= 6.8 \pm 0.8$ cm, $N = 5$)

and 11 minnows (mean $\pm$ SE $= 6.5 \pm 0.17$ cm, $N = 11$) to make the skin extract solutions. We killed each fish by a blow to the head, then carefully filleted the skin from each flank. Skin fillets were measured, then placed in chilled dechlorinated tap water. The total area of skin collected was 68.44 cm^2 and 66.97 cm^2 for cichlids and minnows, respectively. To simulate a predator attack we macerated the skin fillets with a conventional hand-held blender. The solution was filtered through polyester wool to remove scales, connective tissue, and suspended tissue particles. Dechlorinated water was added to bring the total volume of each skin extract to 32 ml. We made 64 sponge blocks of each odor type. Each sponge block was infused with 0.5 ml of dechlorinated water, cichlid skin extract, or minnow skin extract. Therefore, cichlid and minnow cues were of equivalent strength, based on 1.0 cm^2 of skin extract per sponge for each species cue. We froze the sponges at -20°C until needed. Conspecifics exhibit an alarm reaction to skin extract that has been frozen for both fish species used in this experiment (Lawrence and Smith, 1989; Wisenden and Sargent, 1997).

Of the six holes fished each day, two were assigned water sponges, two were assigned cichlid sponges, and two were assigned minnow sponges. We replaced the sponges with fresh ones every 30 min. We also rotated locations so that each sponge type was used at every location during each test day. We recorded the number of times that the tip-up arms showed a clear tip, i.e., the tip of the arm pulled into the hole, and the number of times the indicator flags were released.

We analyzed the data by using a chi-square goodness of fit test. Conventional wisdom maintains that a chi-square test cannot be used if more than 25% of the cells contain frequencies less than 5. However, this oft-repeated rule of thumb is overly conservative and unnecessary (Haldane, 1943; Snedecor and Cochran, 1980, p. 77).

RESULTS

Over the course of 48 hole-hr of fishing effort, holes with the minnow-sponges received seven predator strikes, of which six released the flag, while holes with the cichlid-sponges and water-sponges received one strike (and flag release) each ($\chi^2 = 8.0$, $P < 0.02$). Tip-ups baited with the scent of injured minnows were more attractive to predators than general chemical cues released by an injured non-Ostariophysan fish ($\chi^2 = 4.5$, $P < 0.05$). Predator attraction to the cichlid cue was not different from water ($P \cong 1$).

The time between replacement of the sponge and strike on the lure varied and did not seem related to treatment type. Lures scented with minnow skin were struck 6–24 min (mean $\pm$ SE $= 14 \pm 6$ min, $N = 4$) after sponge replacement. The single strike on the cichlid-scented lure occurred 4 min after sponge replacement. Strike time was not recorded for the water-scented strike.

DISCUSSION

The first conclusion from this experiment is that chemosensory cues are important in eliciting strike behavior. Because minnows are small fishes and fall prey to a wide variety of predators, it is reasonable to infer that fish large enough to trip the tip-up flag were minnow predators. The second conclusion is that not all scents are equally effective. The strike frequency on the cichlid cue was the same as for the water cue, indicating that general substances released from injured fish (amino acids, proteins, lipids, etc.) do not automatically elicit a strike response from predators. Minnow scent was clearly preferred by predators, consistent with predictions from chemical alarm signaling theory (Smith, 1992; Mathis et al., 1995; Chivers et al., 1996). Therefore, this study provides the first field verification from fish predators of the predator-attraction hypothesis for the evolution of ASCs. The predator species that struck the scented lures could not be observed through the ice; however, Lake Ida is best known for its pike fishery during the ice-fishing season. None of the fish striking the scented lures were landed because the sponge block was on the jig hook. Nevertheless, fish large enough to release the tip-up flag would be large enough to eat a fathead minnow or interrupt a predation event by attempts of piracy or cannibalism.

The precise chemistry of the alarm cue contained in minnow skin cells is not completely understood. A nucleotide, hypoxanthine-3(N)-oxide, has long been thought to be the active ingredient in the alarm cue/predator attractant (unpublished PhD thesis by Argentini in 1976, after Smith 1999). Laboratory (Pfeiffer et al., 1985; Mathis et al., 1995) and field (Brown et al., 2000) tests confirm the biological activity of hypoxanthine-3(N)-oxide.

An alternative explanation for the differences in strike frequency is that predators may have preferred minnow cue because minnow cue is familier to them. Cichlids are tropical fish and allopatric to the fish species in Lake Ida. However, there is no evidence to suggest that the chemical cues released by cichlids should differ qualitatively from those typical of general non-Ostariophysan fishes. Cichlids are in the same order, Perciformes, and ecologically similar to centrarchids (sunfish, bass), which are abundant in Lake Ida. The most likely explanation of the differences in strike frequency in the current experiment is that skin from minnows produces a more potent cue, with perhaps better dispersing properties than an equivalent area of cichlid skin. The presence of ASCs in minnow skin is the most likely cause of this.

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ACQUIRED PREDATOR RECOGNITION BY FATHEAD MINNOWS: INFLUENCE OF HABITAT CHARACTERISTICS ON SURVIVAL

KYRA J. GAZDEWICH and DOUGLAS P. CHIVERS*

Department of Biology
112 Science Place
University of Saskatchewan
Saskatoon, Saskatchewan S7N 5E2, Canada

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Abstract—In this study we conditioned fathead minnows (*Pimephales promelas*) to recognize the odor of a perch (*Perca flavescens*) by exposing them to perch odor coupled with minnow alarm cue. We then staged encounters between the predator and prey in order to assess whether the predator odor training had any effect on survival of the prey. We tested for a survival effect in the presence and absence of shelter. Our results indicate that fish trained with alarm signals to recognize predators gained a survival benefit during staged encounters with a predator and that habitat characteristics influenced the survival of conditioned fish.

Key Words—Predator recognition, predator training, chemical alarm signals, predator–prey interactions, survival, habitat, shelter, fathead minnow, *Pimephales promelas*, yellow perch, *Perca flavescens*.

INTRODUCTION

Alarm signaling is the situation in which an animal that encounters a hazard produces a response, the signal, that could make other individuals aware of danger (Smith, 1992). The signal(s) produced can include the sender's escape response or, alternatively, a chemical, sound, visible movement, or other change that may be detected by the receiver (Chivers and Smith, 1998). Many species of aquatic organisms release chemical alarm signals upon being attacked or captured by a predator (Chivers and Smith, 1998; Mirza and Chivers, 2001).

Chemical alarm signals have been shown to be important in facilitating learned recognition of predation risk by prey animals. For example, Chivers and Smith

*To whom correspondence should be addressed. E-mail: doug.chivers@usask.ca

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(1995a,b) showed that fathead minnows (*Pimephales promelas*) learned the identity of dangerous habitats when they detected alarm signals in conjunction with specific habitat odors. Göz (1941) and Magurran (1989) showed that European minnows (*Phoxinus phoxinus*) learned to recognize the odor of a pike (*Esox lucius*) when the pike odor was paired with a minnow alarm signal. Similar studies have demonstrated that fathead minnows learned to recognize the sight or odor of a pike when the pike cues were paired with a conspecific alarm signal (Chivers and Smith, 1994a,b). Several authors provide similar findings in other fishes (Suboski, 1990; Chivers et al., 1995; Berejikian et al., 1999; Yunker et al., 1999; Mirza and Chivers, 2000) as well as in invertebrates (Wisenden et al., 1997; Rochette et al., 1998).

Despite the numerous studies that have documented the importance of alarm cues in learned recognition of unknown predators, only two studies have directly tested whether prey animals that are conditioned to recognize predators have a higher survival upon encountering the predator (Berejikian et al., 1999; Mirza and Chivers, 2001). The results of these studies are mixed. There is a clear need to conduct live predation trials to assess the validity of the current research paradigm (see review Mirza and Chivers, 2001).

The primary objective of the current study was to test whether fathead minnows that are conditioned to recognize predatory yellow perch (*Perca flavescens*) gain a survival benefit during a staged encounter with the predator. A secondary objective was to examine the importance of specific habitat characteristics in determining whether a survival effect was observed. We conducted live predation trials in both the presence and absence of artificial shelter. Minnows conditioned to recognize predators may seek shelter and hence be able to gain a survival benefit over those minnows that do not have shelter available. However, shelter may conceal the location of the predator; hence minnows that are tested in the presence of shelter may be more vulnerable to attacks by the predator.

METHODS AND MATERIALS

Fish Collection and Maintenance. In the fall of 2000, we collected adult yellow perch from Blackstrap Lake, in central Saskatchewan, Canada. The perch were transported to the University of Saskatchewan and held in 150-liter aquaria at 19°C and on a 14L:10D cycle. Prey animals may recognize an unknown predator that has recently fed on conspecifics (see review Chivers and Mirza, 2001). Consequently, we maintained the perch on a diet of brook stickleback (*Culaea inconstans*) prior to collecting stimuli from them.

Fathead minnows that were naïve to perch were obtained from Briarwood Pond, Saskatoon, Saskatchewan. The minnows were held in a 625-liter holding tank at 17°C and on a 14L: 10D cycle. The minnows were fed daily with commercial fish flakes.

Collection of Test Stimuli. Perch odor was prepared by placing two adult perch (≈15 cm standard length) in a 37-liter aquarium for 12 hr. The aquarium was aerated but not filtered. Perch were not fed in the stimulus aquarium. The water was frozen in 60-ml portions, the quantity of perch odor used for each trial.

Skin extract was obtained from five adult fathead minnows that were killed by a single blow to the head, in accordance with the guidelines set by the Canadian Council on Animal Care. Minnow skin fillets (total = 15 cm^2) were placed in 100-ml of chilled glass-distilled water. The skin was homogenized, filtered through filter floss to remove any visible particles, and diluted in distilled water to make a final volume of 150 ml. The extract was frozen in 10-ml lots, and 5 ml were used for each trial. The procedure was repeated to create a "control" extract from six green swordtails (*Xiphophorus helleri*). Swordtails were used as a control because they are phylogenetically distant and allopatric from minnows.

Experimental Protocol. Groups of five adult minnows (*ca.* 4.0−5.0 cm standard length) were placed into separate 37-liter aquaria (50 × 25 × 30 cm) that were filled with 20 liters of dechlorinated tap water. Each aquarium was supplied with an airstone and its sides were covered to prevent disturbance by an observer. The minnows were allowed to acclimate for approximately 22 hr and then were exposed to either perch odor paired with minnow skin extract (experimental treatment) or perch odor paired with swordtail skin extract (control treatment). The perch odor and skin extracts were introduced via a plastic tube. We used a syringe to remove 60 ml of tank water via the tube and retained it. We then slowly injected this into the tube, followed by the 5 ml of skin extract. The stimuli were flushed into the tanks with the 60 ml of tank water. The stimulus was added at a rate of approximately 1 ml/sec. We predicted that minnows would learn to recognize perch odor based on its association with conspecific skin extract, and hence should have a higher survival upon subsequently encountering a perch.

The day following the conditioning trials we removed the test fish from their tanks and conducted the survival trials. We tested the survival of trained and nontrained minnows during staged encounters with perch (*ca.* 15 cm standard length) in 400-liter test aquaria (180 × 45 × 45 cm). Test trials were completed in both the presence and absence of shelter; hence we used a 2 × 2 design, crossing predator training with shelter. Shelter was simulated by hanging 72 strands of twine from each of two pieces of floating Styrofoam measuring 45 × 60 cm. This shelter resembles submergend vegetation in that it visually obstructs the environment but allows the fish to move through it. For treatments done without shelter, two plain square pieces of Styrofoam were placed on the top of the tank to keep the light conditions consistent. Twelve perch were each tested four times, once under each of the different treatment conditions, for a total of 48 trials. The order of treatments was randomized.

Prey animals may recognize any unknown predator that has recently fed on conspecifics of the prey (reviewed by Chivers and Mirza, 2001). Consequently,

prior to each test, the perch were fed stickleback for a minimum of three days and then were starved for three days. Perch were placed into test aquaria at least 2 hr before the start of the trial. Each trial began when the group of five fathead minnows was placed into the test aquaria with the perch. Minnows were introduced into the tank at the maximum possible distance from the perch. For each trial, we recorded the number of minnows surviving after 1 hr. Each aquarium was drained and washed between trials to ensure no alarm cues were present from the previous trial.

We used a series of Wilcoxon signed rank tests to assess effects of the conditioning treatments and shelter treatments on minnow survival (Siegel and Castellan, 1988). The family-wise error rate was assessed and controlled using the modified Bonferroni test following Keppel (1982). The modified Bonferroni test specifies that corrections to the family-wise error rate be introduced only when the number of comparisons exceeds $k - 1$, where k is the number of treatments (Keppel, 1982). In this experiment, there were a total of four treatments. Since the analysis was restricted to four preplanned comparisons, the rejection probability (P) was set at 0.0375 for each comparison (Keppel, 1982).

RESULTS

Both predator training and the presence of shelter had an influence on minnow survival. In the presence of weeds, there was no difference between the survival of minnows conditioned with conspecific extract versus swordtail extract ($T = 23$, $N = 9$ corrected for ties, $P > 0.95$; Figure 1). In contrast, in the absence of weeds, minnows conditioned with conspecific extract survived longer than those conditioned with swordtail extract ($T = 28$, $N = 7$ corrected for ties, $P = 0.016$; Figure 1).

There was no difference in survival for swordtail-conditioned minnows in the presence versus absence of weeds ($T = 22$, $N = 8$ corrected for ties, $P = 0.641$; Figure 1). Minnows conditioned with conspecific extract did not survive longer in the absence of weeds than in the presence of weeds ($T = 25.5$, $N = 7$ corrected for ties, $P = 0.062$; Figure 1).

DISCUSSION

Our results show that fathead minnows can be conditioned to recognize the odor of a perch and that this recognition can translate into increased survival during encounters with the predator. The learned recognition occurs after a single pairing of perch odor with minnow alarm cue. Previous studies have documented that fathead minnows can learn the identity of pike cues in a similar manner (Chivers and Smith, 1994a,b). Complimentary results have been found in several

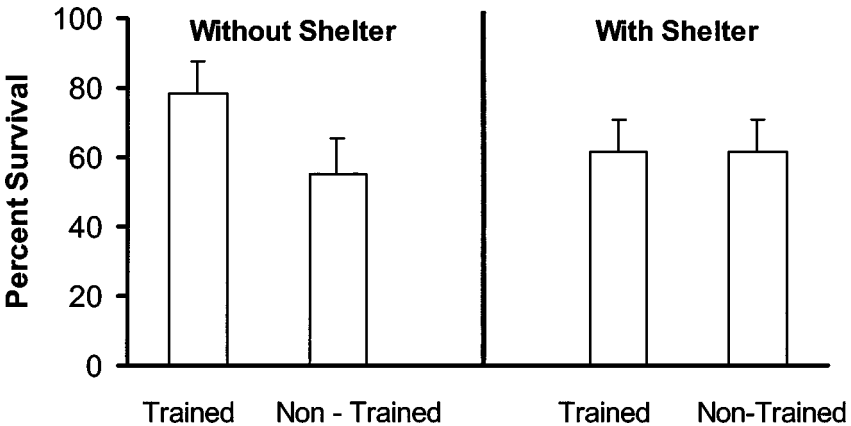


FIG. 1. Mean (+SE) percent survival of trained (conditioned with conspecific skin extract) and nontrained (conditioned with swordtail skin extract) minnows in the presence and absence of shelter during staged encounters with perch.

other species, but whether the conditioning effect translates into increased survival remains untested in most systems.

In our study, we conditioned the prey to recognize the odor of the predator but not the sight of the predator. As such, our results provide empirical evidence of the survival consequences of chemically mediated antipredator responses. Additional studies should examine whether prey conditioned to recognize the sight of a predator gain a survival benefit, and if so, whether the magnitude of the survival effect is dependent on visual versus chemical conditioning.

Only two other studies have tested the survival consequences of conditioning with chemical alarm cues. Mirza and Chivers (2000) showed that juvenile brook charr (*Salvelinus fontinalis*) conditioned to recognize predatory chain pickerel (*Esox niger*) had a greater probability of escaping attacks than nontrained fish. Berejikian et al. (1999) conditioned juvenile chinook salmon (*Oncorhynchus tshawytscha*) to recognize cutthroat trout (*Oncorhynchus clarki*) and then staged encounters between the predator and prey. They observed no survival effect in their first experiment and in their second experiment found that trained fish had a higher survival but that the rearing environment had a significant influence on the result. Fish that were raised in a complex environment, with both overhead and submersed structure, and then conditioned to recognize the trout did not survive better than nontrained controls when stocked in a natural stream. In contrast, fish that were raised in a barren tank environment and then conditioned to recognize predators had higher survival than those that were not trained. Berejikian et al. (1999) speculated that the absence of a survival effect in fish raised in a complex environment may result from the fact that fish from such an environment have a

greater propensity to enter cover, the location where predatory cutthroat trout are most likely to occur.

The results of Berejikian et al. (1999) demonstrate the importance of considering the rearing environment when assessing the potential survival benefit of alarm cue conditioning. The rearing environment may have influenced minnow survival in our study. We collected minnows from Briarwood Pond, a stormwater retention pond with little weed cover. We found an effect of predator recognition training in the absence of weeds but not in the presence of weeds. It is possible that altering the rearing conditions can influence the outcome. For example, minnows reared in an environment with high weed cover could exhibit different types of antipredator defenses that might result in higher survival in the presence of weeds.

It seems clear that fishes and other prey animals can be conditioned to recognize the characteristics of unknown predators based on a single association of the predator cues with alarm signals (reviewed in Chivers and Smith, 1998). Ours is among only a few studies to test specifically whether prey conditioned with alarm cues to recognize predators gain a survival benefit. Moreover, our study is the first to document that habitat characteristics of the testing environment can influence the survival of predator-conditioned fish. We suggest that future work should examine if and when alarm cue conditioning translates into a survival benefit for prey. This should include conducting studies designed to manipulate the rearing conditions and the habitat conditions under which the predator–prey encounter takes place. Habitat variables that could be manipulated include turbidity of the water, presence and density of shelter, and color and type of substrate. Experiments should also employ predators with different hunting strategies. To consider that the survival of prey is dependent exclusively on particular habitat characteristics may be ignoring the possibility of habitat dependence by the predator. Future experiments should also condition prey to recognize visual and chemical cues of predators both singly and together. We need to conduct live predation trials under various conditions in order to assess the potential importance of alarm cue conditioning under natural conditions.

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IDENTIFICATION OF EPOXYHENICOSADIENE AND NOVEL DIEPOXY DERIVATIVES AS SEX PHEROMONE COMPONENTS OF THE CLEAR-WINGED TUSSOCK MOTH *Perina nuda*

SADAO WAKAMURA,[1,*] NORIO ARAKAKI,[2] HIROYUKI YAMAZAWA,[3] NAOTO NAKAJIMA,[3] MASANOBU YAMAMOTO,[3] and TETSU ANDO[3]

[1]*Laboratory of Insect Behavior*
National Institute of Agrobiological Sciences (NIAS)
Ohwashi, Tsukuba 305-8634, Japan

[2]*Okinawa Prefectural Agricultural Experiment Station*
4-222 Sakiyama, Naha 903-0814, Japan

[3]*Graduate School of Bio-Applications and Systems Engineering (BASE)*
Tokyo University of Agriculture and Technology
Koganei, Tokyo 184-8688, Japan

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Abstract—Four EAG-active components (A–D) were found in the solvent extract of virgin females of the clear-winged tussock moth, *Perina nuda*. The most abundant component (B, ca. 250 ng/female) was identified as (3*Z*,6*S*,7*R*,9*Z*)-6,7-epoxyhenicosa-3,9-diene by GC-MS analyses of the extract, chemical derivatization, and comparative chiral HPLC. Minor components also elucidated were (3*Z*,9*Z*)-*cis*-6,7-epoxyicosa-3,9-diene, (A); (3*R*,4*S*,6*S*,7*R*,9*Z*)-3,4-6,7-diepoxyhenicos-9-ene, (C); and its 3*S*,4*R*,6*S*,7*R* isomer, (D); with amounts of 0.4, 5, and 8 ng/female, respectively. Component B showed weak attractiveness to male moths in the field. The attractiveness was significantly enhanced by addition of component(s) C and/or D. No males were captured with either the antipode of component B or its mixtures with the minor components. In this field test, noctuid *Hypocala rostrata* males were also attracted with the synthetic *P. nuda* pheromone.

Key Words—Sex pheromone, *Perina nuda*, Lymantriidae, (3*Z*,6*S*,7*R*,9*Z*)-6,7-eopxyhenicosa-3,9-diene, (3*R*,4*S*,6*S*,7*R*,9*Z*)-3,4-6,7-diepoxyhenicos-9-ene, (3*S*,4*R*,6*S*,7*R*)-3,4-6,7-diepoxyhenicos-9-ene, sex attractant, *Hypocala rostrata*, Noctuidae.

*To whom correspondence should be addressed. E-mail: swaka@affrc.go.jp

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INTRODUCTION

The tussock moth *Perina nuda* (Fabricius) (Lepidoptera: Lymantriidae) is distributed in the Ryukyu Islands (Japan), Taiwan, Malaysia, and India (Inoue, 1982; Azuma, 1996). Adults show remarkable sexual dimorphism. The female has a yellowish body and wings similar to those of the tussock moth species of the genus *Euproctis*. The male has clear wings and a blackish body like those of the cicada.

In Lymantriidae, the sex pheromone chemicals that have been identified are diverse and include hydrocarbons, ketones, epoxides, and esters with and without methyl branches and unsaturation (see Arn et al., 1992, 1997; Gries et al., 1997a,b; Wakamura et al., 2001a,b). In the case of the genus *Euproctis*, different chemicals have been identified from different species. Moreover, no common component has been found (Wakamura et al., 2001a). The sex pheromone component(s) of *P. nuda* are also interesting in terms of pheromone diversity.

Larvae of this insect feed on leaves of the Malayan banyan, *Ficus micromorpha* L.f. ("Gajumaru" in Japanese) (Inoue, 1982; Azuma, 1996). In Okinawa, *P. nuda* larvae have occasionally defoliated the Malayan banyan, especially the horticultural variety that lines streets (Azuma, 1996). Furthermore, human contact with the urticating hair of the caterpillar can cause skin irritation. In Okinawa, insecticide has been sprayed against this species on urban Malayan banyans, but it is often ineffective against severe defoliation.

Chemical identification of the sex pheromone component(s) of *P. nuda* can also improve protection of the Malayan banyan by detecting early moth occurrence that will probably be followed by severe defoliation. This report deals with the identification of sex pheromone components and attractiveness of the synthetic compounds in the field.

METHODS AND MATERIALS

Insects. P. nuda pupae were collected from a horticultural variety of the Malayan banyan found on the streets of Naha, Okinawa on January 14, 2000. Last-instar larvae of this species pupate on the upper surface of the leaves by spinning rough silk cocoons, and the pupae are easy to collect at the outbreak site with forceps. Pupae were treated with 1% sodium hypochlorite solution to wash away the cocoon silk and larval urticating hair, in order to avoid risk of skin irritation. Pupae were sexed and maintained at 25°C and 16L:8D in separate containers until emergence in the laboratory.

Extraction. Solvent extracts of sex pheromone glands were obtained from 1-day-old virgin females at around 30 min after lights-on, since calling behavior was frequently observed at this time. The tip containing the pheromone gland was

excised from the female abdomen with fine tweezers and soaked in hexane (5–10 tips/0.5 ml) for ca. 15 min. This was filtered through a small wad of cotton to obtain an extract. Tips were rinsed with the same volume of hexane twice, and the rinses were added to the extract.

Column Chromatography. Column chromatography was carried out on 200 mg of Florisil (100–200 mesh, Floridin Co.) or silica gel (particle size 75–150 μm, Wako gel, C-200, Wako Pure Chemical Ind. Co., Osaka, Japan) in a Pasteur pipet. Substances were eluted successively with 1 ml each of hexane and 5%, 20%, and 50% ether in hexane.

Gas Chromatography (GC) and Electroantennographic Detection (EAD). GC analyses were conducted on a Hewlett-Packard (HP) 5890II gas chromatograph equipped with a flame ionization detector (FID). An HP-1 or HP-INNOWax fused silica column (15 m $\times$ 0.25 mm ID $\times$ 0.25 μm film thickness) was used at a column head pressure at 55 kPa. Injection was made directly into the capillary column through a cool on-column injector programmed at oven temperature plus 3°C. Temperature program (TP) for the column oven was 50°C for 1 min, 50°C to 150°C at 20°C/min, 150°C to 260°C at 5°C/min, and then final temperature for 5 min [abbreviation for this TP: 50(1)-20-150(0)-5-260(5)]. An electroantennographic (EAG) response was obtained simultaneously with the FID recording. The electroantennographic detector (EAD) was set up according to Struble and Arn (1984), with a minor modification in the column split system.

Coupled Gas Chromatography–Mass Spectrometry (GC-MS). GC-MS analyses were conducted with an HP6890 gas chromatograph interfaced to a Jeol JMS SX-102A double-focusing magnetic sector mass spectrometer (EI mode, 70 eV) and operated with an HP model 715/64 computer. The carrier gas was helium. An HP-1 or HP-Innowax column (15 m $\times$ 0.25 mm ID $\times$ 0.25 μm film thickness; HP) was operated at constant flow mode (1.6 ml/min, ca. 70 cm/sec), with initial column head pressure at 35 kPa. Samples were injected through a cool on-column injector as described above. TP for the column oven was 50(1)-20-150(0)-5-240(5). In certain analyses, a mixture of normal alkanes with even numbers of carbon atoms from icosane (C_{20}) to tricosane (C_{30}) were coinjected for calculation of Kováts retention indices (KI) (Kováts, 1965). These hydrocarbons were eluted at approximate equal intervals from both HP-1 and HP-INNOWax columns.

High-Performance Liquid Chromatography (HPLC). HPLC involved a Jasco PU-980 liquid chromatograph equipped with an integrator (System Instrument Chromatocorder 21J), an RI detector (Labo System RI-98SCOPE), and a Chiralpak AD column (0.46 cm ID $\times$ 25 cm, Daicel Chemical Ind. Co., Ltd., Tokyo, Japan). For the monoepoxide, 0.1% 2-propanol in hexane was used at a rate of 0.45 ml/min, while 0.5% 2-propanol in hexane was used at the same flow rate for the diepoxide. Injection was made through a Rheodyne 7125 loop injector as 10 μl of hexane solution.

Diimide Reduction. Polyenyl compounds were partially or completely hydrogenated according to the method described by Yamaoka et al. (1976). A solution of a polyenyl compound concentrated to about 10 μl in a 1-ml taper-bottomed vial was combined with 10 μl each of hydrazine and hydrogen peroxide solution in ethanol (N_2H_4: 0.3 ml of hydrazine hydrate in 10 ml of ethanol, H_2O_2: 0.04 ml of 31% H_2O_2 in 10 ml of ethanol). The mixture was maintained at 65°C for 1 hr for complete hydrogenation or 55°C for 1 hr for partial hydrogenation. For some other analyses, the reaction was quenched by the addition of 100 μl of 1% HCl solution, and the mixture was immediately extracted with 200 μl of hexane three times. The hexane extract was passed through a short column of ca. 0.1 g of powdered Na_2SO_4 and then concentrated to ca. 10 μl.

Dimethyl Disulfide (DMDS) Adducts. Since yields of direct DMDS derivatization of epoxymonoene were low at the nanogram scale in preliminary experiments, epoxymonoenes were reduced with $LiAlH_4$ before being subjected to DMDS derivatization. About 200 μl of 1 mmol/ml $LiAlH_4$ solution in ether was added to the solution of test material in ca. 100 μl of THF at 0°C, and the mixture was stirred at room temperature overnight. After the reaction had ceased, 100 μl of 1% HCl was added, and the mixture was extracted with 400 μl of hexane three times. Extracts were combined, passed through a layer of powdered Na_2SO_4 (ca. 100 mg), and chromatographed on 200 mg of silica gel in a Pasteur pipet. The fraction eluted with 1.0 ml of 15% ether in hexane was collected after elutions with 1.0 ml each of hexane and 5% ether in hexane.

As in the method described by Buser et al. (1983), 20 μl of DMDS and a very small crystal of iodine were added to ca. 10 μl of hexane solution of the test material in a 100-μl capillary tube with one end sealed. The other end was then sealed, and the capillary was kept at 55°C for 2 hr. After the addition to 100 μl of 5% $Na_2S_2O_3$ solution in distilled water, the products were extracted with 100 μl of hexane three times. The extracts were combined and concentrated to ca. 4 μl, and 0.6 μl of the solution was injected through a cool on-column injector into the GC-MS equipped with an HP-1 column in which the TP was 50(1)-25-200(0)-5-270(5).

Chemicals. *cis*-6,7-Epoxyhenicosane and its *trans*-isomer were obtained from (*Z*)-henicos-6-ene and its *E* isomer, respectively, by oxidation with *m*-chloroperoxybenzoic acid (MCPBA) in dichloromethane. (*Z*)-Henicos-6-ene was synthesized from tetradecyl tosylate and 1-heptyne by an acetylene coupling reaction in HMPA and subsequent hydrogenation over Lindlar catalyst as in Hendry et al. (1975). A part of the *Z* isomer was isomerized to (*E*)-henicos-6-ene by heating in a sealed tube at 60°C for 12 hr with 2,2′-azobis(2-methylpropionitrile) and thiophenol in benzene as in Sgoutas and Kummerow (1969). Geometric isomers were purified by chromatography on SiO_2 impregnated with 16.7% of $AgNO_3$ to $>$99% purity.

(3*Z*,9*Z*)-*cis*-6,7-Epoxyhenicosa-3,9-diene (**1b**) was synthesized from the corresponding (3*Z*,6*Z*,9*Z*)-triene by oxidation with MCPBA (Ando et al., 1993), and pure enantiomers were separated by successive achiral and chiral preparative HPLC (Qin et al., 1997). (*Z*)-*cis*-3,4-*cis*-6,7-Diepoxyhenicos-9-ene (**2**) was prepared by further oxidation of **1b** with MCPBA. Separation of the resulting positional isomers and stereoisomers was also accomplished by preparative HPLC (Yamamoto et al., 1999; Yamazawa et al., 2001).

Dose-Response Relations of EAG Responses. Dose–response curves were measured with the GC-EAD system. A single antenna was used for a series of measurements that ranged from low to high dosages of synthetic compound or extract. Since considerable variation was observed in raw EAG voltage when different antennae were used, the EAG responses were normalized to 1.0 at 10 ng of the 6*S*,7*R* isomer of **1b** (6*S*-**1b**), which was injected immediately after a series of measurements from low to high concentrations.

Field Attraction. A tent-shaped sticky trap (white type, SE trap of Sankei Chem. Co., Kagoshima, 29 × 32 × 8 cm high) with a 24-cm × 30-cm sticky plate was used for field attraction. Synthetic chemicals were applied to the terminal ca. 1 cm^2 area of a 1-cm × 4-cm piece of filter paper (Toyo No. 2, Toyo Roshi Kaisha, Tokyo) as ca. 50 μl of hexane solution. Immediately after the solvent evaporated, the paper pad was suspended about 2 cm above the sticky plate in the trap at about 07:00 hr (attraction of *P. nuda* males occurred around 08:00–09:00 hr). A filter paper pad of the same size was treated with the same volume of hexane as an unbaited control. Trap catches were counted in the afternoon, and the used filter paper pads were removed. Traps were placed on steel rods about 1 m above the ground at 5-m intervals. Trap locations were exchanged in every repetition to cancel out any possible effect of trap locations.

For one test, blends of synthetic chemicals were impregnated in rubber septa (gray halo-butyl isoprene blend elastomer septa, 8 mm outside diam., 19 mm high, West Co., Singapore) by applying ca. 0.3 ml of the hexane solution into the depression. Rubber septum lures were continuously used from late October to early December. Trap locations were exchanged at three- or four-day intervals. Trap catches were counted at intervals of one to three days and pooled for one trap per week, resulting in six replications.

Trap data (X) were transformed to $\sqrt{X + 0.5}$ and subjected to two-way layout ANOVA where zero data (mean = 0) were omitted. The means were ranked by Tukey's method when ANOVA was significant at the 5% level (Figures 6–8 below). Means accompanied by the same letter are not significantly different, and the same letter was given to the zero data when the confidential range of the mean contained zero ($\sqrt{0 + 0.5}$).

Field tests were conducted in Naha, Okinawa, from late October to early December 2000. The population level of *P. nuda* became considerably lower than

expected from observation of the trend of the previous season (September 1999 to January 2000). We were unable to collect larvae or pupae for virgin female traps or to evaluate the effect of the minor component **1a**.

RESULTS

GC and GC-EAD Analyses of the Pheromone Extract. When the accumulated crude extract from 100 virgin females was concentrated to ca. 50 μl and 1 female equivalent (FE)/0.5 μl of the extract was injected into the GC-EAD system, one weak and three distinct EAG-active components (A–D) were observed together with a number of FID peaks (Figure 1). The remaining crude extract was chromatographed on the Florisil column. Components A and B were eluted with 5% ether in hexane (5% fraction), and components B, C, and D were eluted with 20% ether in hexane (20% fraction) (Figure 1). A trace amount of component B in the 20% fraction was probably due to a tailing effect. In separate GC analyses, the amounts in the extracts were estimated to be 0.4, 250, 5, and 8 ng/FE for components A, B, C, and D, respectively, by comparison of FID peak size of 100 ng of tetracosane as an internal standard.

Identification of Components A and B. About 0.3 FE of the 5% fraction was injected into the GC-MS to measure mass spectra, and two corresponding peaks were observed. The mass spectrum for component B (Figure 2B) showed a molecular ion at *m*/*z* 306 (relative intensity 12.9%, base ion: *m*/*z* 67), and diagnostic fragment ions were observed at *m*/*z* 290 (M−16, 4.3%) and *m*/*z* 288 (8.2%, M−18), which suggested an unsaturated epoxide. The mass spectra of the product by complete hydrogenation with diimide at 65°C for 1 hr showed a molecular ion at *m*/*z* 310 (7.7%, base ion: *m*/*z* 83) and fragment ions at *m*/*z* 294 (M−16, 6.3%) and 292 (M−18, 1.1%), which indicated that the component was an epoxide (e.g., McLafferty, 1973). The diagnostic ions at *m*/*z* 239 ($[\overset{\diagup\,\diagdown}{\mathrm{CH}\text{—}\mathrm{CH}}\,\mathrm{C_{14}H_{29}}]^+$, 86%) and *m*/*z* 113 ($[\mathrm{C_5H_{11}}\,\overset{\diagup\,\diagdown}{\mathrm{CH}\text{—}\mathrm{CH}}\,]^+$, 38%) indicated the functional group at the 6,7 position. The KI value on the polar column (HP-INNOWax) was 2499, which was similar to that for the authentic *cis*-6,7-epoxyhenicosane (2498) but apparently different from that for the *trans* isomer (2475). Component B was, thus, considered to be *cis*-6,7-epoxyhenicosadiene. Since the mass spectra of the original component B showed two mass unit smaller fragment ions at *m*/*z* 237 and *m*/*z* 111 than the hydrogenated compound, the double bonds were considered to be located on either side of the epoxide group (Ando et al., 1993).

To determine the position of the double bonds in component B, a 5% fraction (ca. 10 FE) was treated with diimide at 55°C for 1 hr to obtain a mixture that

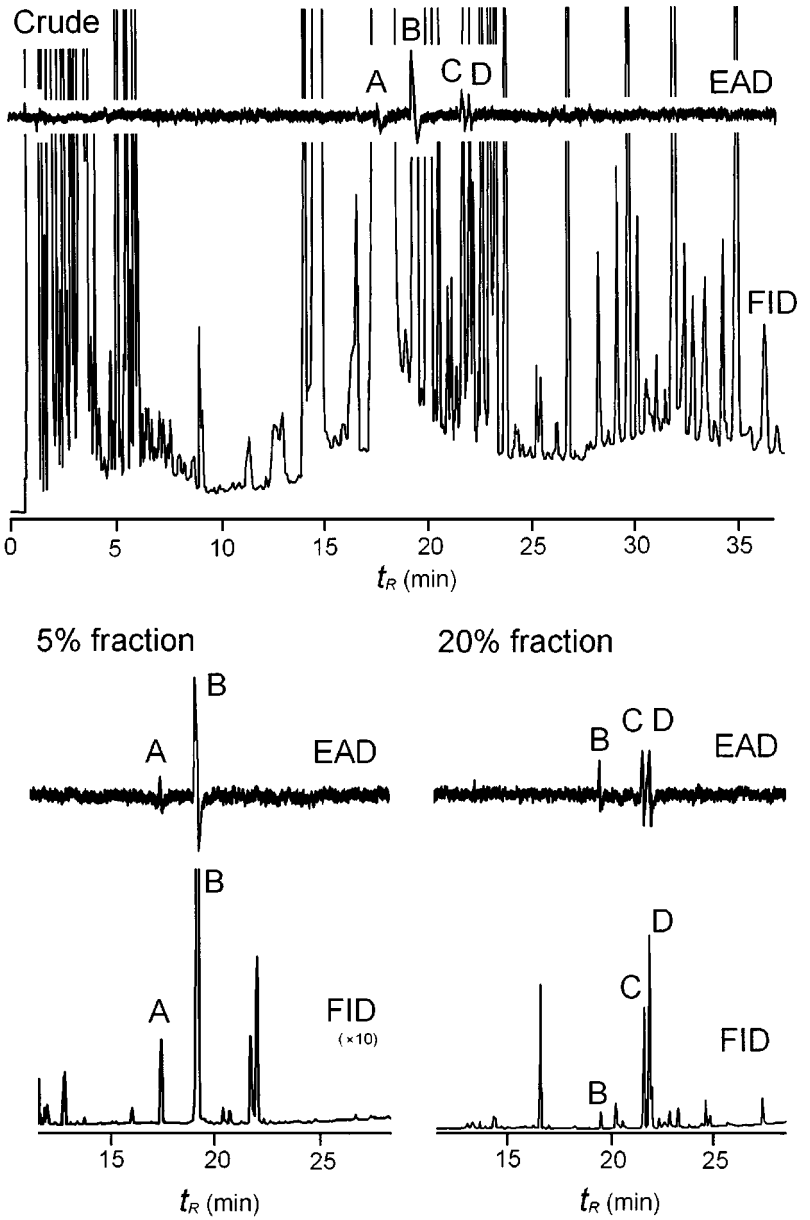


FIG. 1. GC-EAD chromatograms of crude extract of virgin *P. nuda* females and 5% and 20% ether-in-hexane fractions from Florisil column chromatography.

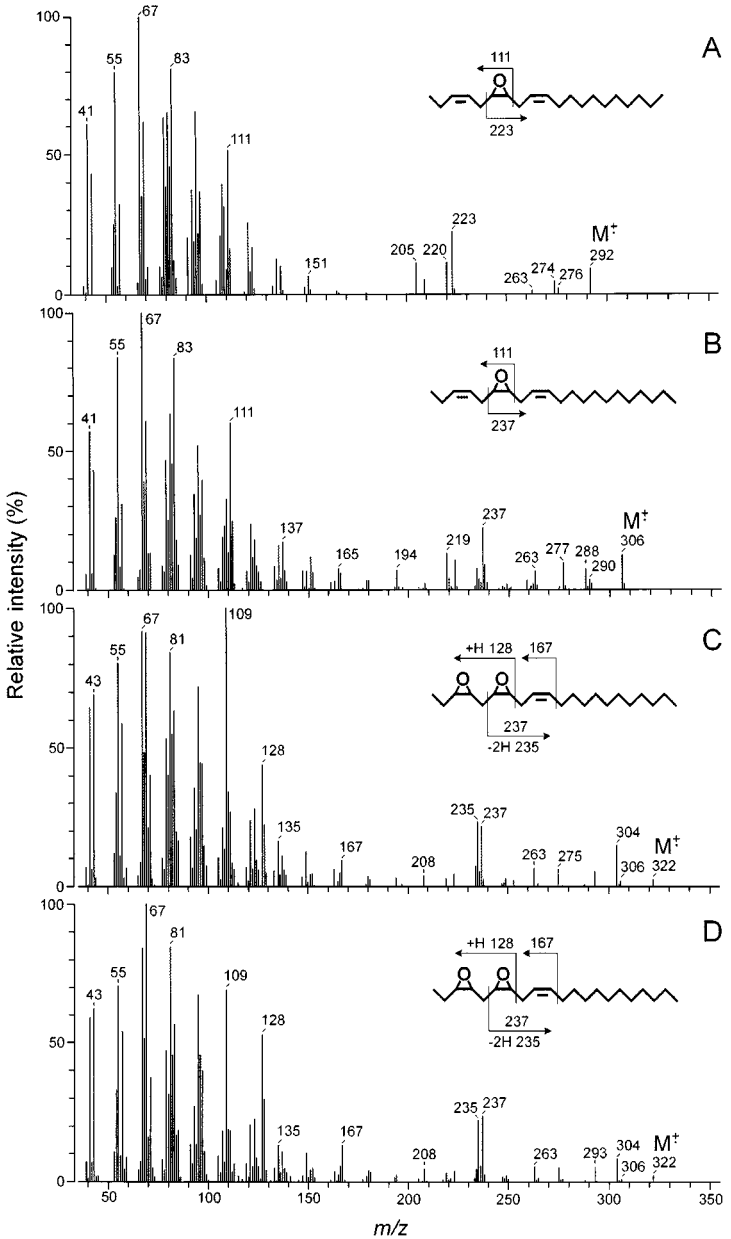


FIG. 2. EI mass spectra of EAG-active components A–D in the extracts of virgin *P. nuda* females. JEOL JMS SX-102A at 70 eV.

contained monounsaturated epoxides. These epoxides were then reduced with $LiAlH_4$ to corresponding alcohols. The unsaturated alcohols were treated with DMDS, and the DMDS adducts were analyzed by GC-MS. Two expected adducts were detected: the mass spectrum of one product showed the molecular ion at *m*/*z* 404 (17.9%) and a diagnostic fragment ion at *m*/*z* 215 {$[CH_3(CH_2)_{10}CHSCH_3]^+$, 100%}, which indicated that one olefin position was at the 12th carbon atom from the end (9th carbon from the opposite end). The partner ion expected at *m*/*z* 189 was not observed, probably because of the effect of the alcohol group. The other product also gave a molecular ion at *m*/*z* 404 (15.0%, base *m*/*z* 75) and the diagnostic ion at *m*/*z* 89 [$(CH_3CH_2CHSCH_3)^+$, 30%], which indicated a double bond at the third carbon. This implied that the original component B included a 3,9-diene system.

Almost all the epoxides that have been identified as sex pheromone components from lepidopterous insects possess a *Z* configuration (see Arn et al., 1992, 1997). The only known exception is posticlure, (6*Z*,9*Z*,11*S*,12*S*)-11,12-epoxyhenicosa-6,9-diene, a sex pheromone component of *Orgyia postica* (Walker) (Lepidoptera: Lymantriidae) (Wakamura et al., 2001b). As expected, component B and the authentic (*Z*,*Z*)-*cis*-6,7-epoxyhenicosa-3,9-diene (**1b**) showed almost identical mass spectra and KI values on both nonpolar HP-1 (KI 2220) and polar HP-INNOWax (KI 2555–2556) columns. Therefore, component B was considered to be an epoxydiene **1b**, although there remains a slight possibility of another geometric isomerism.

The mass spectrum of minor component A (Figure 2A) showed a molecular ion at *m*/*z* 292 (10.1%, base: *m*/*z* 67), and a similar series of fragment ions to those for component B, but the *m*/*z* values in the high mass region were 14 mass units smaller than those of component B. These similarities suggested that component A is (*Z*,*Z*)-*cis*-6,7-epoxyicosa-3,9-diene (**1a**), an analog of component B.

Identification of Components C and D. Components C and D in the 20% fraction of the Florisil column showed almost the same mass spectra (Figure 2C,D). In both spectra, the ion that appeared at *m*/*z* 322 was considered to be a molecular ion. Significant fragment ions at *m*/*z* 306 (M–16) and 304 (M–18) indicated an epoxide group in the molecule. However, the KI values for these components on both nonpolar HP-1 (KI 2369 and 2387) and polar HP-Innowax (KI 2909 and 2948) columns (Table 1) were larger than expected for monoenyl C_{22} epoxides (KI ~2320 for HP-1, ~2655 for HP-INNOWax). This suggested the presence of an additional polar functional group such as an epoxide.

Based on the homology in the chemical structure of component B, (9*Z*)-*cis*-3,4-*cis*-6,7-diepoxyhenicos-9-ene (**2**) and (3*Z*)-*cis*-6,7-*cis*-9,10-diepoxyhenicos-3-ene were the most likely candidates for components C and D. In the GC-EAG analyses, diastereomers of **2** (racemic mixtures) evoked more intensive EAG response than the other candidates (Figure 3B). In the GC-MS analyses of the

TABLE 1. KI VALUES OF COMPONENTS C AND D AND SYNTHETIC *cis,cis*-DIEPOXYHENICOSENES[a]

Compounds	HP-1	HP-Innowax
EAG-active components in VF extract		
Component C	2369	2906
Component D	2387	2945
Synthetic diepoxyhenicosenes[b]		
9*Z*(3*S**6*R**)	2369	2908
9*Z*(3*S**6*S**)	2387	2946
6*Z*(3*S**9*R**)	2389	2953
6*Z*(3*S**9*S**)	2397	2966
3*Z*(6*S**9*R**)	2371	2904
3*Z*(6*S**9*S**)	2396	2954

[a] KI values were measured with a GC-MS system. See text for GC conditions.

[b] Synthetic diepoxyhenicosenes are presented by abbreviation: e.g., 9*Z*(3*S**6*R**) is a racemic mixture of (3***S***,4*R*,6*R*,7*S*,9*Z*)-3,4-6,7-diepoxyhenicos-9-ene and its antipode, the 3*R*,4*S*,6*S*,7*R*,9*Z* isomer.

synthetic diepoxy compounds that include (6*Z*)-*cis*-3,4-*cis*-9,10-diepoxyhenicos-6-ene (Yamazawa et al., 2001), two diastereomers of **2** showed similar mass spectra and identical KI values to components C and D (Table 1). Components C and D were, thus, estimated to be two diastereomers of **2**.

Stereochemistry of Components B–D. To determine chirality of component B, ca. 20 FE (5 μg) of 5% fraction was injected into the HPLC equipped with a chiral column. Component B was eluted as a single peak at $t_R = 15.98$ min. This value was almost identical to that for the 6*S*,7*R* enantiomer, but smaller than that for the 6*R*,7*S* enantiomer (16.67 min), as shown in Figure 4. The identity was confirmed by coinjection of 20 FE of 5% fraction and 10 μg of the racemic mixture. Component B was, thus, considered to be (3*Z*,6*S*,7*R*,9*Z*)-6,7-epoxyhenicosa-3,9-diene [(6*S*)-**1b**]. This conclusion was also supported by the GC-EAG analyses (Figure 3A). The dose–response curve of the enantiomers of (6*S*)-**1b** was equivalent to that of the 5% fraction of the virgin female extracts, while the enantiomer (6*R*)-**1b** elicited significantly less antennal response.

The 20% fraction of another 140 FE of extracts was injected onto HPLC, and eluates were fractionated at 0.50-min intervals from $t_R = 20.00$ min. In the RI monitor, small and unclear peaks were observed at around $t_R = 22.33$ min and $t_R = 24.46$ min (Figure 5A). The racemic mixture of two diastereomers of **2** (ca. 20 μg) was also fractionated under the same conditions. Three peaks were observed in the RI monitor: the (3*S*,4*R*,6*R*,7*S*) isomer (3*S*6*R*)-**2** at $t_R = 20.19$ min, the (3*S*,4*R*,6*S*,7*R*) isomer [(3*S*6*S*)-**2**] at $t_R = 22.33$ min, and the (3*R*,4*S*,6*S*,7*R*)

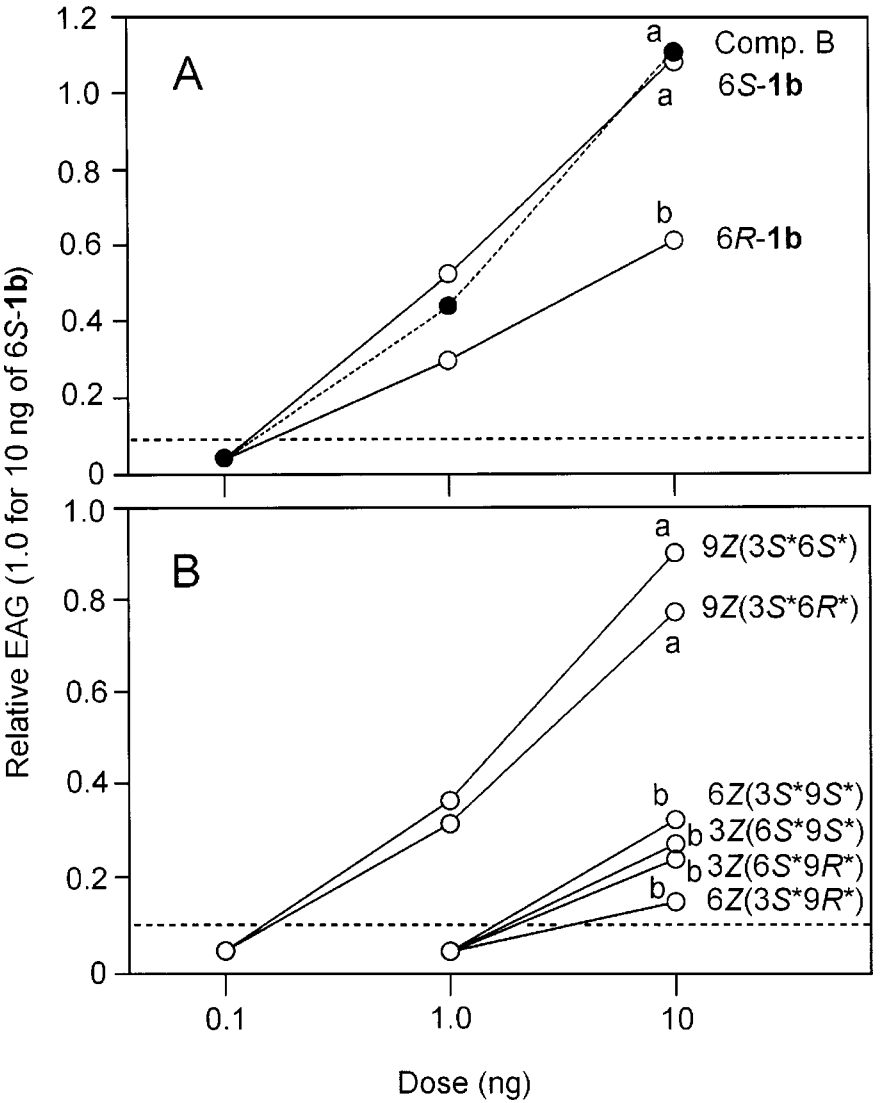


FIG. 3. EAG responses to authentic (3*Z*,6*S*,7*R*,9*Z*)-6,7-epoxyhenicosa-3,9-diene [(6*S*)-**1b**], its enantiomer [(6*R*)-**1b**], and component B in the virgin female extract (A) and to authentic diepoxymonoene isomers (racemic mixture) (B). For abbreviations, see footnote to Table 1. EAG values are normalized to 1.0 for 10 ng of (6*S*)-**1b**. Values indicated are means for six replications for (6*S*)-**1b**, (6*R*)-**1b**, 9*Z*(3*S**6*S**) [= (3*S*6*S*)-**2** + (3*R*6*R*)-**2**] and 9*Z*(3*S**6*R**) [= (3*S*6*R*)-**2** + (3*R*6*S*)-**2**]. The other isomers had three replications. Values accompanied by the same letter are not significantly different by Tukey's test ($P > 0.05$). Horizontal dashed lines indicate noise levels.

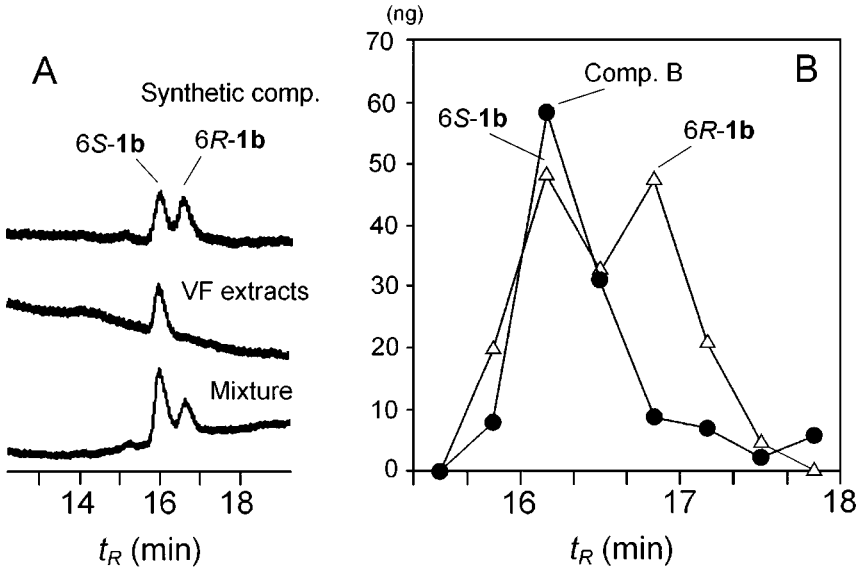


FIG. 4. HPLC analyses of component B in the 5% fraction of the virgin female extracts and synthetic *cis*-6,7-epoxyhenicosa-3,9-diene (**1b**: racemic mixture) (A) and quantitative GC analyses of the HPLC fractions (B). Solid circles and open triangles represent the amounts of compounds in the HPLC fractions of the 5% fraction of virgin female extracts and synthetic **1b**, respectively.

isomer [(3*R*6*S*)-**2**] and (3*R*,4*S*,6*R*,7*S*) isomer [(3*R*6*R*)-**2**] at $t_R = 24.56$ min as a slightly broad peak.

In order to determine the amounts of the components in the fractions, GC analyses were done on the HP-1 column (Figure 5B). Before injection, 100 ng of docosane was added to the HPLC fractions of the 20% fraction as an internal standard and concentrated to ca. 100 μl. Two hundred nanograms of docosane was added to the HPLC fraction of the authentic components. Components C and D were detected in the HPLC fractions from $t_R = 24.00$ to 25.50 min and from $t_R = 22.00$ to 23.00 min, respectively. Authentic (3*S*6*R*)-**2** and its enantiomer were detected in the fractions from $t_R = 20.00$ to 21.50 min and from $t_R = 24.00$ to 25.50 min, respectively. In contrast, authentic (3*S*6*S*)-**2** and its enantiomer were observed in the fractions from $t_R = 22.00$ to 23.50 min and from $t_R = 24.50$ to 25.50 min. Components C and D were, thus, confirmed to be (3*R*6*S*)-**2** and (3*S*6*S*)-**2**, respectively.

Field Attraction of P. nuda. Field tests were conducted at Naha, Okinawa, from late October to early December 2000. Male catches were observed mainly from 08:00 to 09:00 hr. Apparently, more *P. nuda* males were captured with

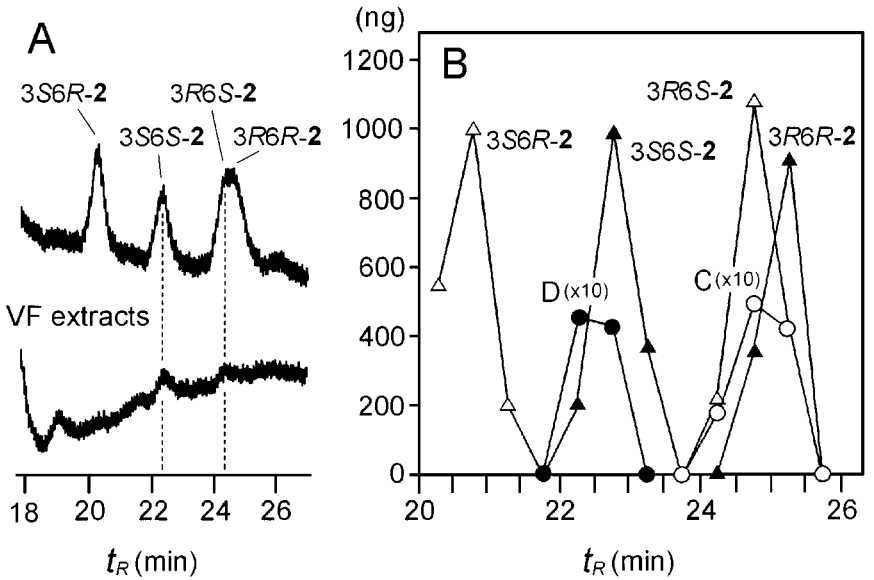


FIG. 5. HPLC analyses of components C and D in the 20% fraction of the virgin female extracts and a mixture of authentic isomers of diepoxyhenicosene (**2**) (A) and quantitative GC analyses of the HPLC fractions (B)

500:20:30 μg/rubber septum blends of (6*S*)-**1b**, (3*R*6*S*)-**2**, and either (3*S*6*S*)-**2** or (3*R*6*R*)-**2** than with the other blends, although the difference was not always significant (Figure 6). A small number of males was captured with (6*S*)-**1b** alone, but there were no captures with the blends containing (6*R*)-**1b**. To determine which minor diepoxide component is synergistic, 50:2 or 50:3 μg/paper pad blends of (6*S*)-**1b** and one stereoisomer of diepoxide components (**2**) were examined. Significantly more males were captured when either (3*R*6*S*)-**2** or (3*S*6*S*)-**2** was blended with (6*S*)-**1b**, whereas blends of (6*S*)-**1b** with either (3*S*6*R*)-**2** or (3*R*6*S*)-**2** were equivalent to (6*S*)-**1b** alone (Figure 7A). However, the synergistic effect of single diepoxide components did not increase when the two diepoxide components were blended together in a subsequent test (Figure 7B). In this test, no males were captured with the single diepoxide components (3*R*6*S*)-**2** or (3*S*6*S*)-**2**. A small number of males were captured with (6*S*)-**1b** alone (Figure 8A,B). These observations confirmed that (6*S*)-**1b** and either one or both of the minor diepoxides, (3*R*6*S*)-**2** and (3*S*6*S*)-**2**, are the sex pheromone components of *P. nuda.*

Field Attraction of Noctuid Hypocala rostrata. H. rostrata (Fabricius) (Lepidopera: Noctuidae) males were frequently captured with the traps baited with the synthetic compounds loaded on rubber septa (Figure 8). Captures were similar to those of *P. nuda* (Figure 6): they apparently occurred with formulations

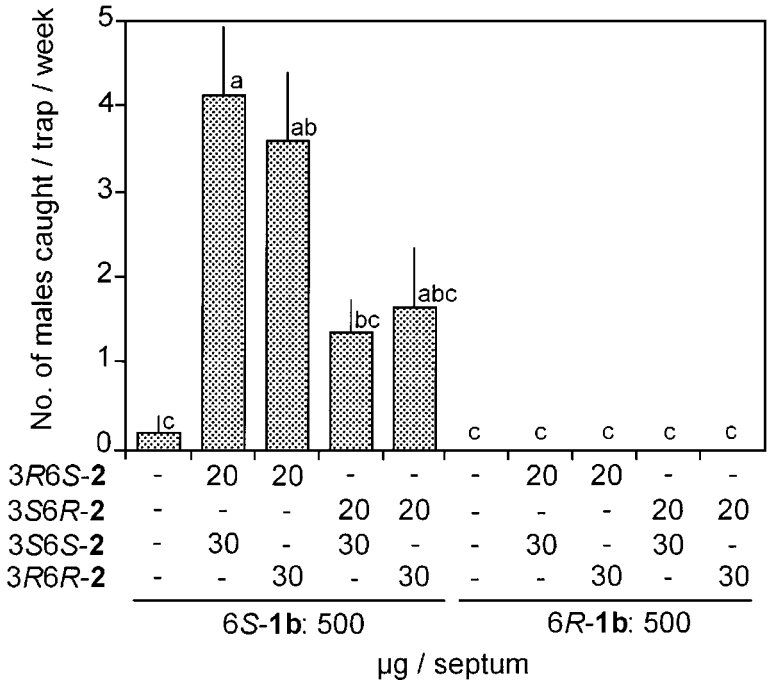


FIG. 6. Captures of male *P. nuda* with blends of stereoisomers of (3*Z*,9*Z*)-*cis*-6,7-epoxyhenicosa-3,9-diene (**1b**) and (9*Z*)-*cis*-3,4-*cis*-6,7-diepoxyhenicos-9-ene (**2**). Values are means and standard errors (SE) of pooled catches for one trap for six weeks (= 6 replications) from October 26 to December 2, 2000. Means capped with the same letter are not significantly different ($P > 0.05$; see text for details of statistical analyses). (6*S*)-**1b** and (6*R*)-**1b** are 6*S*,7*R* and 6*R*,7*S* isomers of **1b**, respectively, and (3*R*6*S*)-**2**, (3*S*6*R*)-**2**, (3*S*6*S*)-**2**, and (3*R*6*R*)-**2** are (3*R*,4*S*,6*S*,7*R*), (3*S*,4*R*,6*R*,7*S*), (3*S*,4*R*,6*S*,7*R*), and (3*R*,4*S*,6*R*,7*S*) isomers of **2**, respectively.

that contained (6*S*)-**1b**. Formulations with antipode failed to yield any captures. Captures with (6*S*)-**1b** combined with either (3*R*6*S*)-**2** or (3*S*6*S*)-**2** were greater than with (6*S*)-**1b** alone but the difference was not significant.

DISCUSSION

Four EAG-active components in the extracts of abdominal tips of *P. nuda* females were identified as (3*Z*,9*Z*)-6,7-epoxyicosa-3,9-diene (**1a**), (3*Z*,6*S*,7*R*,9*Z*)-6,7-epoxyhenicosa-3,9-diene [(6S)-**1b**], (3*R*,4*S*,6*S*,7*R*,9*Z*)-3,4-6,7-diepoxyhenicos-9-ene [(3*R*6*S*)-**2**] and its (3*S*,4*R*,6*S*,7*R*,9*Z*) isomer [(3*S*6*S*)-**2**], by means of chemical analyses including GC-MS, chemical derivatization, syntheses, and

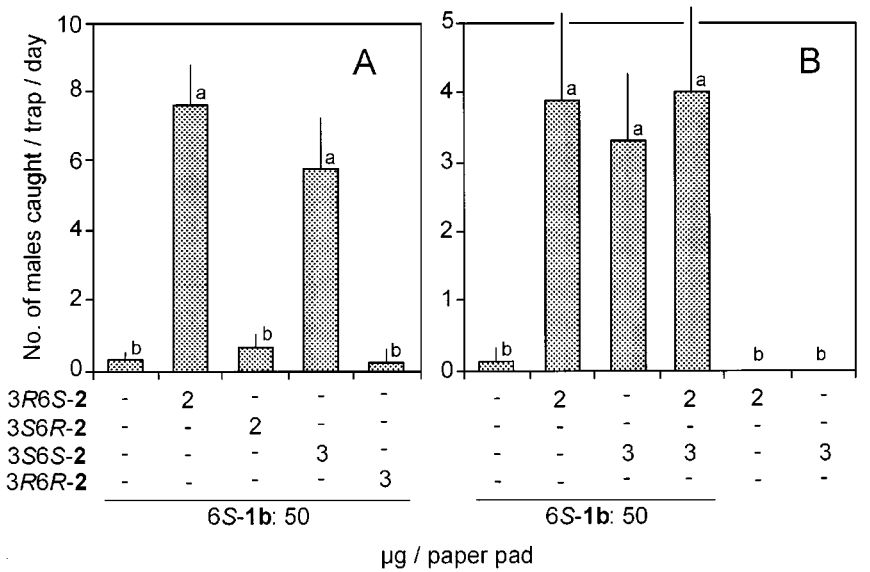


FIG. 7. Captures of male *P. nuda* with blends of (3*Z*,6*S*,7*R*,9*Z*)-6,7-epoxyhenicosa-3,9-diene [(6*S*)-**1b**] and stereoisomers of (9*Z*)-*cis*-3,4-*cis*-6,7-diepoxyhenicos-9-ene (**2**). Values are means and SE for one trap for five days (= 5 replications) for every five days between November 16 and 23 (A) and from November 26 to 30 (B) 2000. For abbreviations of compounds and statistical analyses, see Figure 6 legend.

chiral HPLC. (6*S*)-**1b** was shown to be an essential component for male attraction in the field experiment, and either or both diastereomers of **2** were synergistic (Figures 7 and 8). These C_{21} components were, thus, confirmed to be sex pheromone components of *P. nuda*. However, details of the stereochemistry of the minor C_{20} component **1a** and the attractiveness of blends of synthetic compounds that include enantiomers of **1a** to compete with that of virgin females are still unknown.

Both diastereomers found in the extract of the sex pheromone gland elicited equal male antenna responses (Figure 3B) and equally increased male catch when blended with **1b** (Figure 7B). It is interesting that the two diepoxide diastereomers act neither additively nor synergistically, but the presence of either is equivalent to the presence of both in male attraction. Similar redundancy between enantiomers has been observed in a lymantriid, *Euproctis pseudoconspersa*, in which females release the *R* enantiomer (Ichikawa et al., 1995), and the male antenna show significantly greater EAG responses to the *R* than the *S* enantiomer (Wakamura et al., 1996). In the field, however, slightly more males were attracted with the *R* enantiomer (Japan: Wakamura et al., 1996; China: Zhao et al., 1998).

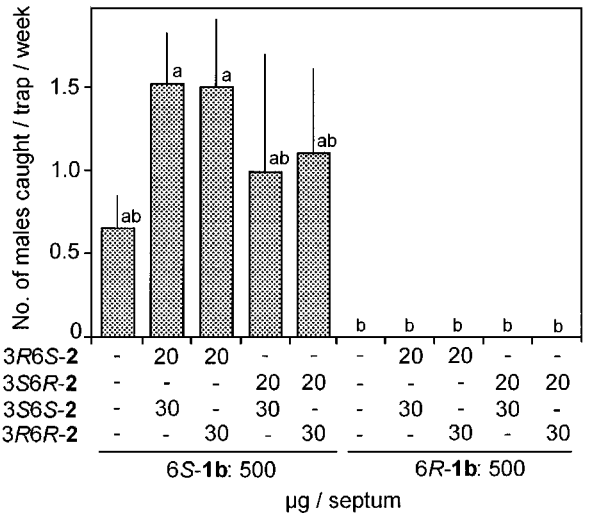


FIG. 8. Captures of male *Hypocala rostrata* with blends of stereoisomers of (3*Z*,9*Z*)-*cis*-6,7-epoxyhenicosa-3,9-diene (**1b**) and (9*Z*)-*cis*-3,4-*cis*-6,7-diepoxyhenicos-9-ene (**2**). Values are means of pooled catches for one trap for six weeks (= 6 replications) from October 26 to December 2, 2000. For abbreviations of compounds and statistical analyses, see Figure 6 legend.

In *Trichoplusia ni* (Hübner) (Noctuidae), a neurophysiological study suggested that redundancy occurs at the receptor level on neighboring dendrites (Todd et al., 1995). In *P. nuda*, chirality of one epoxide group at the 6,7 position in **1b** and **2** seems to be strictly discriminated both in production by the female (Figures 4 and 5) and in response by male antenna (Figures 3, 6, and 7), while that at the 3,4 position does not in either or both sexes. It is also interesting that redundancy might occur in biosynthesis in the female pheromone gland and/or in perception by receptors on male antenna.

Males of a noctuid moth *Hypocala rostrata* (Fabricius) were also captured with the blends containing (6*S*)-**1b** (Figure 8). This species and *P. nuda* probably share (6*S*)-**1b** as a sex pheromone component. Capture of *H. rostrata*, however, occurred during the night (the exact time is unknown) and contrasted to the morning captures of *P. nuda*. The mating time is probably different for these two species. It would be interesting to study whether the diepoxide compounds have any effect on male catches of *H. rostrata*.

The major component (6*S*)-**1b** has been identified as a sex pheromone component from *Tetanolita mynesalis* (Walker) (Noctuidae) (Haynes et al., 1996). In field screening tests conducted in Canada, males of *Idia americalis* (Guenée), *Bleptina caradrinalis* (Guenée) (both Noctuidae), *Xanthorhoe munitata* Hübner, *X.*

abrasaria aquilonaria (Herrich-Schäffer), and *X. ferrugata* (Clerck) (all Geometriidae) were captured with blends containing (6*S*)-**1b** (Millar et al., 1991). In Japan, males of *Paragabora flavemacula* (Oberthür) (Noctuidae) were captured with the corresponding racemic mixture (Ando et al., 1993). Probably this is the first case in which (6*S*)-**1b** was found in families other than Noctuidae and Geomtriidae.

Diepoxide pheromone components were first identified from *Leucoma salicis* L. (Lymantriidae): (3*Z*)-*cis*-6,7-*cis*-9,10-diepoxyhenicos-3-ene (Gries et al., 1997a). The absolute configuration has not been determined. The minor diastereomers (3*R*,6*S*)-**2** and (3*S*,6*S*)-**2** are, therefore, probably newly recognized compounds as sex pheromone components in Lymantriidae as well as in Lepidoptera. The addition of these findings has certainly increased the diversity of known pheromone chemicals in Lymantriidae.

In this study, we used only chiral compounds for the field tests, so the activity of the racemic mixture still needs to be evaluated. Limited amounts of chiral compound compelled us to use small filter paper sheets instead of rubber septa as release devices. In our experience, the filter paper-sheet may release larger amounts of chemicals than rubber septa, but effective duration is too short for monitoring use (effective for only one or two days with *P. nuda*). Daily trap catches seemed several times greater with ca. 50 μg of chemicals treated on filter paper pads (Figures 8) than with 550 μg on rubber septa (Figure 6), although direct comparison was inadequate. Nevertheless, this suggested that more captures would occur when larger amounts of chemicals are loaded on rubber septa.

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2-METHYL-(*Z*)-7-OCTADECENE: SEX PHEROMONE OF ALLOPATRIC *Lymantria lucescens* AND *L. serva*

GERHARD GRIES,[1,*] PAUL W. SCHAEFER,[2] REGINE GRIES,[1] YI-BIN FAN,[3] YASUTOMO HIGASHIURA,[4] and BAN TANAKA[5]

[1]*Center for Environmental Biology, Department of Biological Sciences*
Simon Fraser University
Burnaby, British Columbia V5A 1S6, Canada

[2]*United States Department of Agriculture, Agricultural Research Service*
Beneficial Insects Introduction Research Laboratory
Newark, Delaware 19713

[3]*Taiwan Forestry Research Institute*
Taipei 100, Taiwan

[4]*Ecology Laboratory, Tokyo University of Pharmacy and Life Science*
Horinouchi, Hachioji, Tokyo 192-0392, Japan

[5]*Toyota Yahagi River Institute*
2-19, Nishimachi, Toyota, Aichi 471-0025, Japan

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Abstract—Our objective was to identify the sex pheromone of *Lymantria lucescens* and *Lymantria serva* (Lepidoptera: Lymantriidae), whose larvae defoliate, respectively, *Quercus* spp. in temperate regions and *Ficus* spp. in the subtropics. Coupled gas chromatographic-electroantennographic (GC-EAD) detection analyses of pheromone gland extracts revealed one EAD active compound produced by female *L. lucescens* and by female *L. serva*. This was identified as 2-methyl-(*Z*)-7-octadecene (2me-*Z*7-18Hy) by retention index calculations on DB-5, DB-23, and DB-210 columns and by comparative GC-mass spectrometric (MS) and GC-EAD analyses of the insect-produced candidate pheromone and synthetic 2me-*Z*7-18Hy. In field experiments, traps baited with 2me-*Z*7-18Hy captured male *L. lucescens* near Toyota City, Japan, and male *L. serva* in Taipei, Taiwan. Allopatric distribution of *L. lucescens* and *L. serva* seems to allow both species to use the same sex pheromone without compromising its specificity.

Key Words—Lymantriidae, *Lymantria lucescens*, *Lymantria serva*, sex pheromone, reproductive isolation, 2-methyl-(*Z*)-7-octadecene, (+)-disparlure, (−)-disparlure, (+)-monachalure, (−)-monachalure, (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane, (7*S*,8*R*)-*cis*-7,8-epoxy-2-methyloctadecane, (7*R*,8*S*)-*cis*-7,8-epoxy-octadecane, (7*S*,8*R*)-*cis*-7,8-epoxy-octadecane.

* To whom correspondence should be addressed. E-mail: gries@sfu.ca

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INTRODUCTION

Lymantria lucescens (Lepidoptera: Lymantriidae) is a temperate species present on the islands of Hokkaido, Honshu, and Kyushu of Japan (Inoue, 1957), and in Korea (Nam and Kim, 1981; P.W.S., unpublished). Larvae feed on oak trees, *Quercus* spp. (Fagaceae). *Lymantria serva* is a subtropical species known to occur in Taiwan (Kishida, 1992), southern China (Collenette, 1933; Zhao, 1982), including Hong Kong (R. Kendrick, personal communication 1999), Nepal (Kishida, 1995), and India (Gupta, 1992). In Taiwan, it appears to have two generations per year. Larvae feed on several species of *Ficus* (Moraceae).

Schaefer and Kishida (1999) captured male *L. lucescens* in sticky traps baited with (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane [(+)-disparlure], (7*R*,8*S*)-*cis*-7,8-epoxy-octadecane [(+)-monachalure], and 2-methyl-(*Z*)-7-octadecene (2me-*Z*7-18Hy), sex pheromone components of the nun moth, *L. monacha* (Bierl et al., 1975; Klimetzek et al., 1976; Grant et al., 1996; Gries et al., 1996). Either or all of these compounds could be pheromone components of *L. lucescens*. No attractant was known for *L. serva.*

A moderate population density of *L. serva* in Taipei, Taiwan (1996–1999) and an outbreak population of *L. lucescens* near Toyota City, Japan (2000) allowed us to field collect larvae and to laboratory-rear them to adults for pheromone analyses. We report identification and field testing of the sex phermone of *L. lucescens* and *L. serva.*

METHODS AND MATERIALS

Experimental Insects. Between 1996 and 1999, *L. serva* larvae or pupae were collected on *Ficus microcarpa* in a schoolyard in Taipei, Taiwan and either hand-carried or shipped to the USDA Quarantine Facility in Newark, Delaware, USA. From these shipments, two mated females were reared and their egg masses obtained. Because neonate larvae fed only reluctantly on ornamental *Ficus* spp., they were offered and accepted lichens from local Newark tree trunks before feeding on *Ficus microcarpa*, which they preferred over *F. elastica*. Despite these diet adjustments, larvae developed slowly, and only a few specimens reached the adult stage.

Between May 27 and 28, 2000, near Toyota, Japan, 326 intermediate stage larva *L. lucescens* were collected on foliage of oak trees, including *Quercus variabilis*, *Q. serrata*, and *Q. glauca* (all new host records). Larvae of the gypsy moth, *Lymantria dispar*, and pink gypsy moth, *L. mathura*, were feeding concomitantly on the same trees. Larvae of *L. lucescens* were reared to adults at Tokyo University of Pharmacy and Life Science, and in the Quarantine Facility in Newark.

Extraction and Analyses of Pheromone. Abdominal tips with pheromone glands of calling, 2- to 3-day-old females were removed and extracted for

15–30 min in HPLC grade hexane. Ampuoles with the supernatant of pheromone extracts and male pupae were sent by courier to Simon Fraser University.

Aliquots of <0.5 female equivalents (FE) (*L. serva*) and >1 FE (*L. lucescens*) of pheromone gland extracts were analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975) with a Hewlett Packard (HP) 5890A gas chromatograph equipped with a fused silica column (30 m $\times$ 0.25 or 0.32 mm ID) coated with either DB-210, DB-5, or DB-23 (J&W Scientific, Folsom, California, USA). GC-EAD analyses were replicated twice (*L. serva*) and three to five times (*L. lucescens*) with each of these fused silica columns. GC-mass spectra of synthetic or insect-produced compounds in full-scan electron ionization mode were obtained from a Varian Saturn II Ion Trap GC-MS fitted with the above-referenced DB-5 column.

Field Experiments. Field experiments were conducted in 2000 in the Botanical Garden of Taipei (25°2′N, 121°30′E) and in a mixed oak forest near Toyota (35°8′N, 137°11′E). Delta-type traps made from two-liter milk cartons (Gray et al., 1984) were coated with Tanglefoot (The Tanglefoot Company, Grand Rapids, Michigan, USA), and suspended from trees $\sim$2 m above ground in complete randomized blocks with trap spacings of 15–20 m. Traps were baited with gray sleeve stoppers (The West Company, Lionville, Pennsylvania, USA) impreganted with test chemicals in HPLC grade hexane.

Experiments 1 and 2 and 3–8 were conducted with *L. serva* and *L. lucescens*, respectively. Although 2me-*Z*7-18Hy was the only EAD-active candidate pheromone component in both species, experiments also assessed other conceivable candidate pheromone components in combination with 2me-*Z*7-18Hy or singly, including disparlure (the corresponding epoxide of 2me-*Z*7-18Hy; experiments 1, 2, 7), 2-methyl-(*Z*)-7-heptadecene (2me-*Z*7-17Hy, the lower homolog of 2me-*Z*7-18Hy; experiment 3), and (*Z*)-7-octadecene (*Z*7-18Hy, the straight chain equivalent of 2me-*Z*7-18Hy; experiment 4). Both (+)- and (−)-monachalure, which are components in the volatile blend of *L. monacha* (Gries et al., 1996), were tested as potential synomonal repellents for *L. lucescens* (experiment 6). Experiment 5 tested increasing quantities of 2me-*Z*7-18Hy, and experiment 8 tested diel periodicity of response by male *L. lucescens* to pheromone-baited traps.

Various treatments in several experiments did not attract any male moths. Despite transformation, trap catch data were not normally distributed and were, thus, analyzed by nonparametric analyses of variance (Friedman's test) followed by comparison of means by Bonferroni (Dunn) *t* test (Zar, 1984; SAS/STAT, 1988). In all analyses, $\alpha = 0.05$.

RESULTS AND DISCUSSION

GC-EAD analyses of pheromone extract of female *L. serva* and *L. lucescens* (Figure 1) revealed a single component that consistently elicited responses from

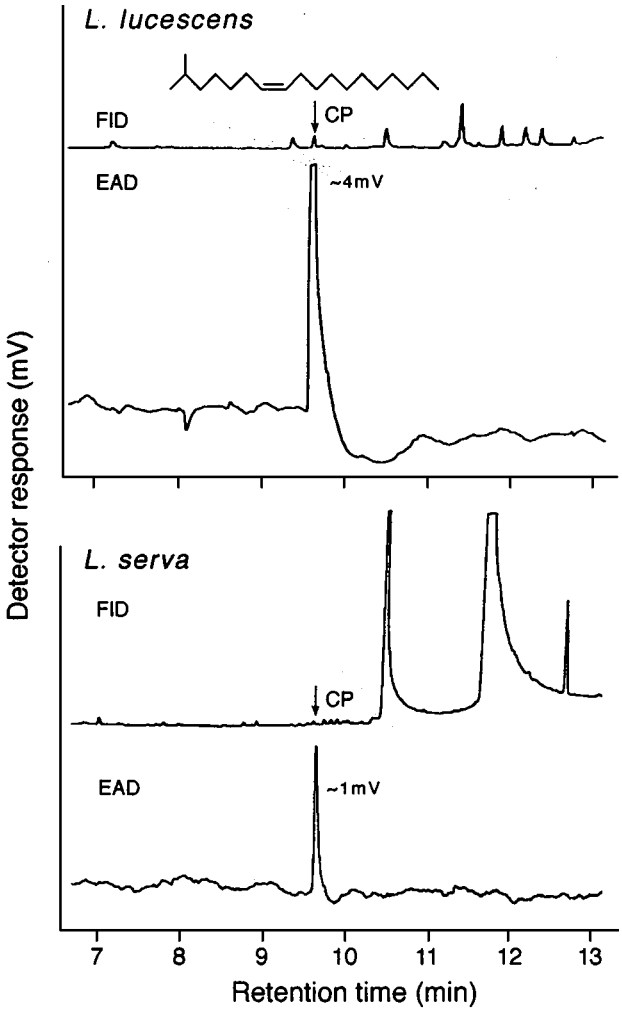


FIG. 1. Top: Flame-ionization detector (FID) and electroantennographic detector (EAD: male *L. lucescens* antenna) responses to >1 female equivalent (FE) of pheromone gland extract from female *L. lucescens*. Bottom: FID and EAD (male *L. serva* antenna) responses to <0.5 FE of pheromone gland extract from female *L. serva*. Chromatography: splitless injection; injector and FID detector: 240°C; DB-23 column; temperature program: 100°C (1 min), then 10°C/min to 200°C (5 min).

male antennae. Retention indices (Van den Dool and Kratz, 1963) and a mass spectrum of this compound in gland extracts of female *L. lucescens* indicated that it was 2-methyl-(*Z*)-7-octadecene (2me-*Z*7-18Hy). Identical retention and mass spectrometric characteristics as well as comparable EAD activity of the candidate pheromone (CP in Figure 1) and of synthetic 2me-*Z*7-18Hy confirmed this structural assignment.

In field experiments 1 and 2 with *L. serva* (Figure 2), 2me-*Z*7-18Hy, but not disparlure, attracted males. The attractiveness of 2me-*Z*7-18Hy was not affected by addition of either (+)-, (−)-, or (+)- plus (−)-disparlure (Figure 2, experiment 2). In experiments 3–5 with *L. lucescens* (Figure 3), 2me-*Z*7-18Hy attracted males, whereas 2me-*Z*7-17Hy or *Z*7-18Hy were not behaviorally active (experiments 3, 4). Increasing doses of 2me-*Z*7-18Hy resulted in increasing attractiveness of the lure (experiment 5). Addition of (+)- monachalure or (+)-disparlure to 2me-Z7-18Hy decreased attractiveness of the lure, whereas addition of (−)-monachalure or (−)-disparlure had no behavioral effect (Figure 4, experiments 6, 7). Male *L. lucescens* were captured in pheromone-baited traps between 20:00 and 23:00 hr (Figure 5, experiment 8).

Laboratory analyses of pheromone extract and field experiments provide conclusive evidence that 2me-*Z*7-18Hy is the single-component sex pheromone of both *L. serva* and *L. lucescens*. In GC-EAD recordings of pheromone extracts and in field experiments, it was the only compound that elicited antennal responses and attracted male *L. serva* and *L. lucescens*.

Considering that 2me-*Z*7-18Hy and (+)-disparlure are synergistic pheromone components in both *L. monacha* from Bohemia, Czech Republic (Grant et al., 1996; Gries et al., 1996) and *L. fumida* from Honshu, Japan (Schaefer et al., 1999), disparlure and other conceivable candidate pheromone components (2me-*Z*7-17Hy, *Z*7-18Hy) were also field-tested, but were found not to be attractive. On the contrary, (+)-disparlure reduced attraction of male *L. lucescens* to 2me-*Z*7-18Hy (Figure 4, experiment 7). We conclude that male *L. lucescens* are repelled by synomonal (+)-disparlure from *L. fumida* during synchronic communication periods (Figure 5), and by synomonal (+)-monachalure from *L. monacha* despite allochronic communication periods of *L. monacha* and *L. lucescens* (Figure 5).

The single-component sex pheromone (2me-*Z*7-18Hy) of *L. lucescens* differs from that of all other congeners and, when tested by itself, did not cross-attract any other lymantriid moth (this study; Schaefer et al., 1999; Gries et al., unpublished). With the specificity of 2me-*Z*7-18Hy, and with synomonal repellency of (+)-disparlure from *L. fumida* and (+)-monachalure from *L. monacha*, male *L. lucescens* may not have experienced selective pressure to evolve the ability to recognize (−)-disparlure as a reinforcing synomonal repellent (Figure 4, experiment 7).

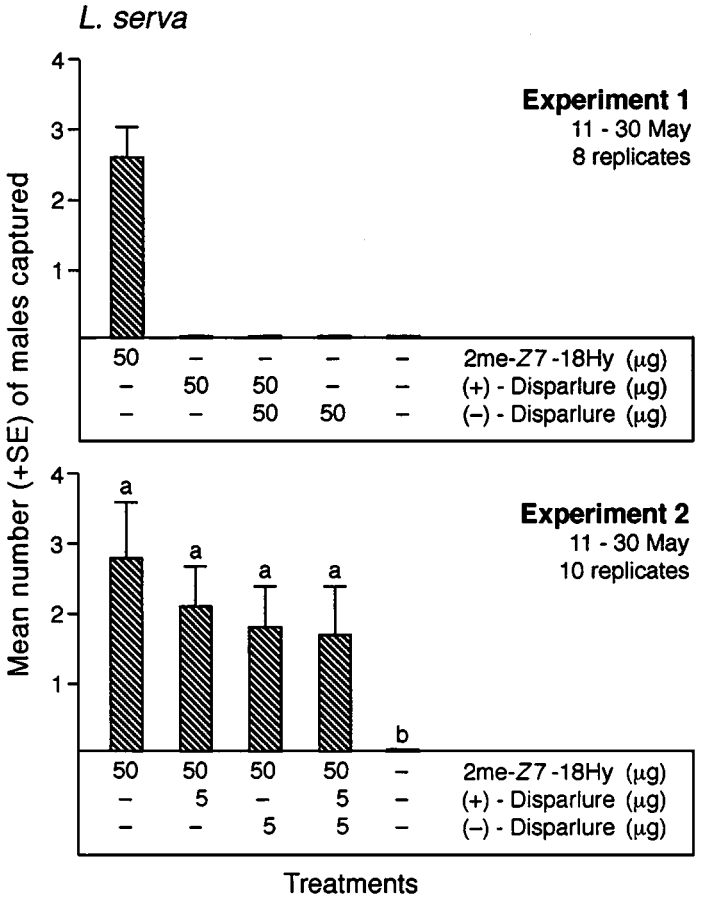


FIG. 2. Captures of male *L. serva* in experiments 1 and 2 in sticky Delta traps baited with test chemicals singly and in combination. Botanical Garden, Taipei, Taiwan. In experiment 1, attractiveness of only one treatment did not warrant statistical analyses of data. In experiment 2, bars with different superscript letters are significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means (Student-Newman-Keuls test) (Zar, 1984), $P < 0.05$.

Allopatric distribution of *L. lucescens* and *L. serva* seems to allow both species to use the same sex pheromone without compromising specificity. Response to sex pheromones from congeners seems to be affected by both the composition of the lymantriid community and communication channels of its member species. Synchronous communication periods of *L. fumida* and *L. lucescens* (Figure 5) may explain the reduced attraction of male *L. lucescens* to pheromonal

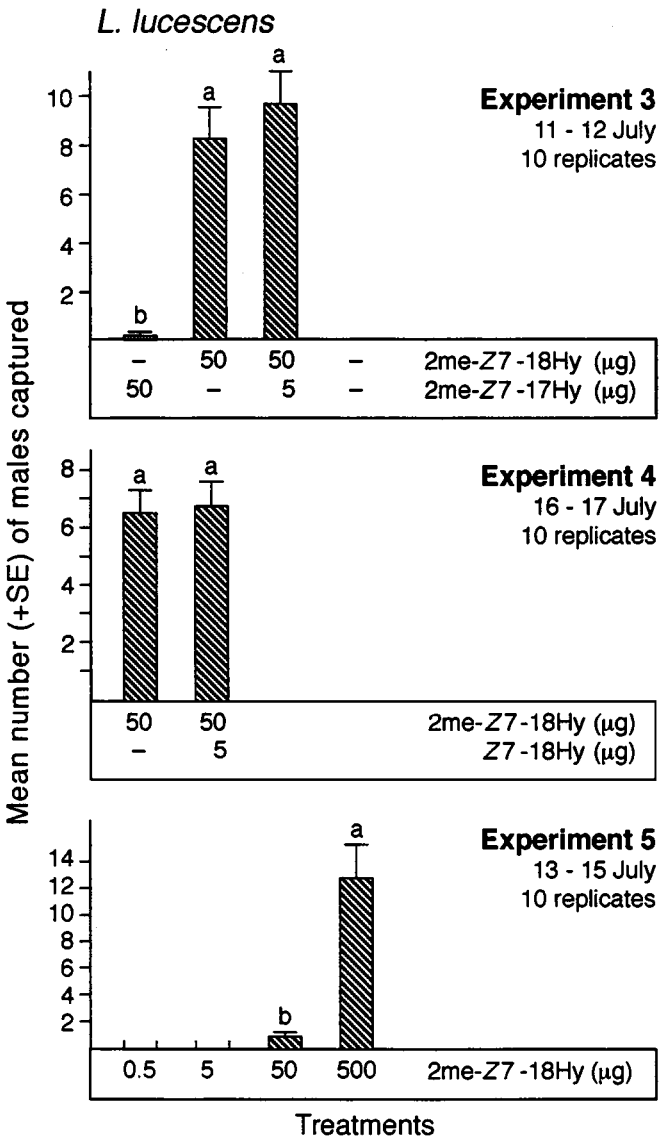


FIG. 3. Captures of male *L. lucescens* in experiments 3–5 in sticky Delta traps baited with candidate pheromone components singly and in combination. Oak (*Quercus* spp.) forest near Toyota City, Honshu, Japan. In each experiment, bars with different superscript letters are significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means (Student-Newman Keul's test) (Zar, 1984), $P < 0.05$.

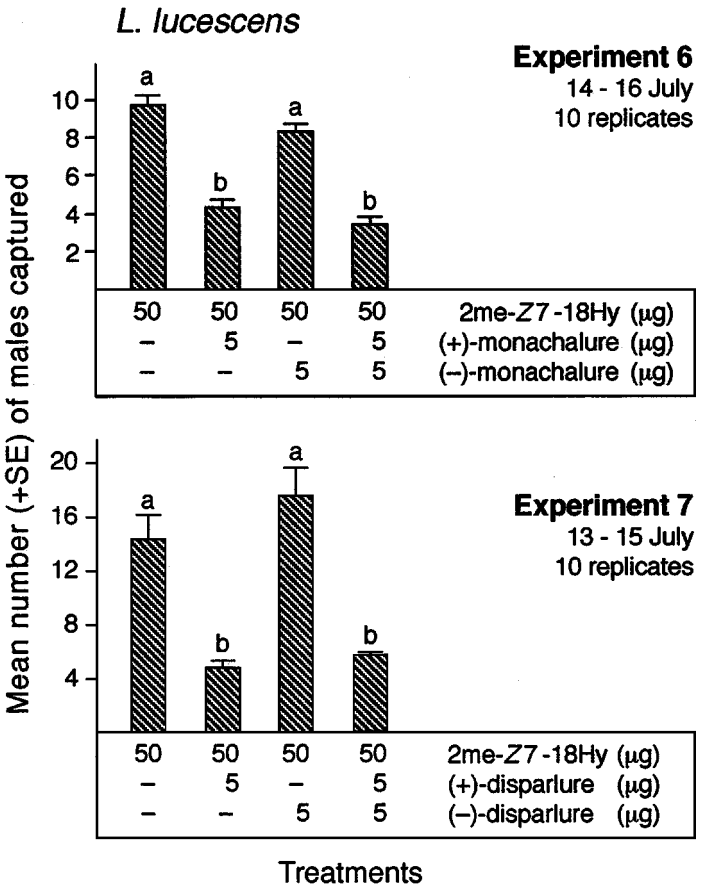


FIG. 4. Captures of male *L. lucescens* in experiments 6 and 7 in sticky Delta traps baited with the pheromone 2me-*Z*7-18Hy singly and in combination with candidate synomonal repellents. Oak (*Quercus* spp.) forest near Toyota, Honshu, Japan. In each experiment, bars with different superscript letters are significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means (Student-Newman Keul's test) (Zar, 1984), $P < 0.05$.

2me-*Z*7-18Hy in the presence of (+)-disparlure (Figure 4, experiment 7), the major pheromone component of *L. fumida* (Schaefer et al., 1999). With no such effect observed in the experiment with *L. serva* (Figure 2), there is not likely a congener in the *L. serva* community in Taiwan that uses (+)-disparlure as a pheromone component within the communication period of *L. serva.* If true, our data support the hypothesis that community composition is a "biology factor" that affects

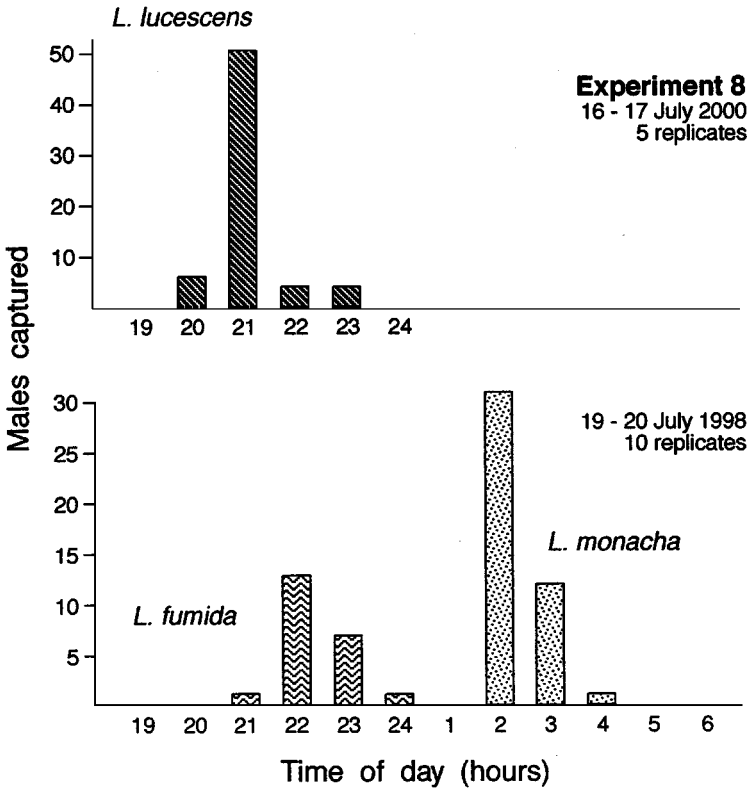


FIG. 5. Top: Hourly recording of captures of male *L. lucescens* in sticky Delta traps ($N = 5$) spaced 50 m apart and baited with 2-methyl-(*Z*)-7-octadecene (500 μg). Oak forest near Toyota, southern Honshu, Japan. Bottom: Representative hourly recording of captures of male *L. fumida* and male *L. monacha* in sticky Delta traps baited with either (+)-disparlure (50 μg) and 2-methyl-(*Z*)-7-octadecene (5 μg) (for *L. fumida*) or with (+)-monachlure (50 μg), (+)-disparlure (5 μg), and 2-methyl-(*Z*)-7-octadecene (5 μg) (for *L. monacha*). Larch forest near the City of Morioka, northern Honshu, Japan. Traps with baits for *L. fumida* ($N = 10$) or *L. monacha* ($N = 10$) were alternated at 15- to 20-m intervals (adapted from Schaefer et al., 1999).

recognition and evolution of intra- and interspecific communication signals in the Lepidoptera (Linn and Roelofs, 1995).

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ACTIVITY OF MALE PHEROMONE OF MELANESIAN RHINOCEROS BEETLE *Scapanes australis*[1]

DIDIER ROCHAT,[2,*] JEAN-PAUL MORIN,[1,2,3] TITUS KAKUL,[4] LAURENCE BEAUDOIN-OLLIVIER,[3,5] ROBERT PRIOR,[4] MICHEL RENOU,[2] ISABELLE MALOSSE,[2] TANYA STATHERS,[4] SEBASTIAN EMBUPA,[4] and SAMSON LAUP[4]

[1]*Work supported by the European Commission under INCO-DC project ERB-IC18CT970199.*

[2]*INRA, Unité de Phytopharmacie et Médiateurs Chimiques*
Centre de Versailles, Route de Saint-Cyr
78026 Versailles Cedex, France

[3]*CIRAD-CP, TA 80/02*
Avenue Agropolis
34398 Montpellier Cedex 5, France

[4]*Cocoa and Coconut Research Institute (CCRI)*
P.O. Box 1846
Rabaul, Papua New Guinea

[5]*CCRI*
P.O. Box 642
Madang, Papua New Guinea

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Abstract—Laboratory and field investigations were carried out to investigate the nature and role of the male pheromone emitted by the Dynast beetle *Scapanes australis* and to develop a mass trapping technique against this major coconut pest in Papua New Guinea. We report the biological data obtained from natural and synthetic pheromone, previously described as an 84:12:4 (w/w) mixture of 2-butanol (**1**), 3-hydoxy-2-butanone (**2**), and 2,3-butanediol (**3**). EAG recordings from natural and synthetic pheromone and a pitfall olfactometer were poorly informative. In contrast, extensive field trapping trials with various synthetic pheromone mixtures and doses showed that **1** and **2** (formulated in polyethylene sachets in 90:5 v/v ratio) were necessary and sufficient for optimum long-range attraction. Beetles were captured in traps baited with racemic **1** plus **2**, with or without a stereoisomer mixture of **3** (2.5- to 2500-mg/day doses). Plant pieces, either sugarcane or coconut, enhanced captures by the synthetic pheromone, which was active alone. Traps with the pheromone caught both sexes in a 3:2 female–male ratio. A pheromone-based mass trapping led to the capture

* To whom correspondence should be addressed. E-mail: rochatd@versailles.inra.fr

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of 2173 beetles in 14 traps surrounding 40 ha of a cocoa-coconut plantation. The captures followed a log-linear decrease during the 125-week trapping program. The role of the male pheromone and its potential for crop protection are discussed.

Key Words—Coleoptera, Scarabaeidae, *Scapanes*, aggregation pheromone, EAG, field trapping, coconut pest.

INTRODUCTION

Scapanes australis Bsdv. (Coleoptera, Scarabaeidae, Dynastinae) is a 4- to 6-cm long horned beetle and a major coconut pest endemic in Papua New Guinea (PNG), the Solomon Islands, and Indonesia (Irian Jaya); (Bedford, 1976; Dechambre, 1995). The adults mine galleries to feed in the bases of fronds where they hide during daytime. Given their size, they frequently destroy the growth point in young coconut palms. On bigger palms, the damage attracts black palm weevils (BPW), *Rhynchophorus bilineatus* (Montr.), which lay eggs. The weevil larvae cause new damage, leading inevitably to the death of the palms. In East New Britain (ENB) province of PNG, the combined *S. australis*–BPW attacks are so common that coconut replanting has become impossible for the last 20 years. Neither chemical nor biological control of *S. australis* has been effective to date because of cost, the risks to human health, and the lack of efficient pathogens and predators (Beaudoin-Ollivier et al., 1999). A prospective way of reducing *S. australis* primary damage is to eliminate the adults by selective trapping with attractants. Great hopes have been placed in this system, for pheromones have been recently identified in the Dynastinae *Oryctes monoceros* (Oliv.) and *Oryctes rhinoceros* (L.) (Gries et al., 1994; Hallet et al., 1995; Morin et al., 1996), which are now used in South-Eastern Asia (Ho, 1996; Chung, 1997). More recently, a particular male calling behavior has been described in *S. australis* (Prior et al., 2000): at nightfall, the males move to the gallery entrance, take up a special position with the abdomen and rear legs raised outwards while the head is in the gallery, and emit a liquid secretion, rhythmically smeared onto the pygidium by crossing the rear legs. In field assays with caged insects on young coconut palms, attraction of both sexes to calling males was evidenced. The attraction was assumed to be elicited by a pheromone from the male secretion. The solid phase microextraction (SPME) associated with a specific protocol allowed the identification of three highly volatile compounds that characterized the male secretion (Rochat et al., 2000a). These were **1**: 2-butanol [= *sec*-butanol; in a 67:33 (*R*)-(−):(*S*)-(+) ratio], **2**: acetoin (= 3-hydroxy-2-butanone), and **3**: 2,3-butanediol [in a 43:17:40 (*R*, *R*)-(−):(*S*, *S*)-(+):*meso* ratio], emitted in a 84:12:4 w/w ratio in the headspace. We examined the pheromone properties of the male secretion and of synthetic **1–3**. The ecological role of the pheromone and its potential for pest control are discussed.

METHODS AND MATERIALS

Synthetic Chemicals

(±)-2-Butanol (>99% GC purity; Aldrich Chemical Co., Gillingham-Dorset, UK), acetoin (>97% GC; crystallized mixture of dimer and monomer; Fluka Chemie AG, Buchs, Switzerland), and (±)-2,3-butanediol (>98% GC; mixture of (2*R**,3*R**) and *meso* forms; Aldrich Chemical Co., Gillingham-Dorset, UK) were used for laboratory and field experiments. Acetoin was heated to melt. A reference 90:5:5 (v/v) **1:2:3** mixture was designed using SPME to reproduce the headspace ratio (84:12:4, w/w) obtained from the male secretion (Rochat et al., 2000a). Ethanol (96%: EtOH) and ethyl acetate (EtAc) (>99% GC; Ajax Laboratory Chemicals, Australia) were used to prepare the kairomone mixtures A–C. A is a 50:50 (v/v) EtOH:EtAc mixture. B is based on a 42:47 (v/v) EtOH:EtAc mixture plus 11% of 14 compounds identified in the volatiles released by sugarcane and coconut (described under reference G in Rochat et al., 2000b). C is a 375:375:50:25:75:25:75 mixture of EtOH, EtAc, hexanol, guaiacol, 2-phenylethanol, phenol, and ethyl propionate. All minor compounds were commercial products as reported in Rochat et al. (2000b).

Collection and Preparation of the Natural Pheromone

Several types of samples were prepared in an initial process to study the pheromone.

Effluvia Collection. Effluvia were collected by passing a stream of purified air over activated charcoal (75 ml/min) into a 1-liter glass flask containing one insect (*S. australis* ssp. *grossepunctatus* (Stern.); CCRI Keravat Station, ENB, PNG). They were adsorbed onto 350-mg Supelpak-2 (Supelco). As it was not possible to maintain a calling male in the flask, collections were carried out in 3-hr sessions on several consecutive evenings, with one male extracted from its gallery while calling. For comparison, effluvia were collected at the same time from flasks containing one female or no insect (control). The adsorbent was rinsed with 1.1 ml dichloromethane. The samples were used in electroantennography.

Wiping Beetle Abdomens with Filter Papers. The filter papers were washed in a Soxhlet apparatus with hexane (Sigma, HPLC grade) prior to use. One paper was held with tweezers and wiped once over the tip of the abdomen of a calling male, then placed immediately in a sealed tube with 1 ml solvent: acetone or dichloromethane (HPLC grade). Samples were taken on several consecutive evenings from calling males artificially settled in coconut plants in the laboratory (PNG). The wiping operations were counted to establish a stimulus concentration: the number of filter papers per microliter of solvent. Although females did not adopt a calling behavior, samples were also taken from them for comparison.

Control samples (unwiped filter papers) were prepared. Two independent series of wipings were performed: one with 40-mm^2 papers on *S. a. grossepunctatus* individuals (CCRI Keravat Station, ENB) and used in electroantennography. The other one with 100-mm^2 papers on *S. a. australis* individuals (CCRI Steward Research Station, Madang) and used in olfactometry.

Capillary Collection of Male Secretion. Because organic extracts obtained by wiping and effluvia collections did not reveal male-specific compounds by gas chromatography (GC) and triggered weak EAG responses, the secretion emitted by 10 calling males (*S. a. grossepunctatus*; laboratory, France) was collected directly in 10-μl capillary glass tubes. These tubes were stored in a tight vial and kept at -12°C during the collection sessions (Rochat et al., 2000a). Male secretion from the various individuals were pooled, stored at -40°C, and used as a stimulus.

Olfactometer Experiments

These experiments were conducted at CCRI Steward Research Station on *S. a. australis* individuals (tested and bait) by using a two-choice pitfall olfactometer (45 cm diameter $\times$ 20 cm high) adapted from Rochat et al. (1991). The pits consisted of 600-ml glass beakers (15 cm high $\times$ 6.5 cm diam.). Beetles, collected from family palm plantations were kept in aerated plastic boxes with sugarcane pieces. Tests lasted 30 min. They were conducted during the 4 hr after nightfall using two olfactometers simultaneously in the laboratory ($27 \pm 1^\circ$C; $70 \pm 5\%$ relative humidity). Coconut stem pieces (10×5 cm) with an artificial gallery were placed in the pits when required. Responses of both sexes ($22 < N < 125$) in the following five experiments were recorded: 1, one male in coconut versus blank control; 2, one male in coconut versus coconut (control); 3, one female in coconut versus blank control; 4, coconut without insect versus blank control; and 5, one filter paper wiped with the male secretion versus an unwiped filter paper (control). Wiping procedures were done before the olfactometer tests and the filter papers were stored in 1 ml acetone at -10°C. The rareness of females found in the coconuts (Prior et al., 2000) explains the lower number of replicates performed with females and the use of some individuals two or three times in certain experiments (Figure 1). A test beetle was responding when it fell into one pit (choice). The repartition of the choices was compared using one-tailed binomial tests at 5%. The calling activity of the bait males was recorded in experiments 1 and 2. The proportions of responding beetles to calling and to noncalling bait males were compared using χ^2 tests.

Electroantennography

Electroantennograms were recorded from *S. a. grossepunctatus* individuals by using the same equipment as described for *O. rhinoceros* (Morin et al., 1996).

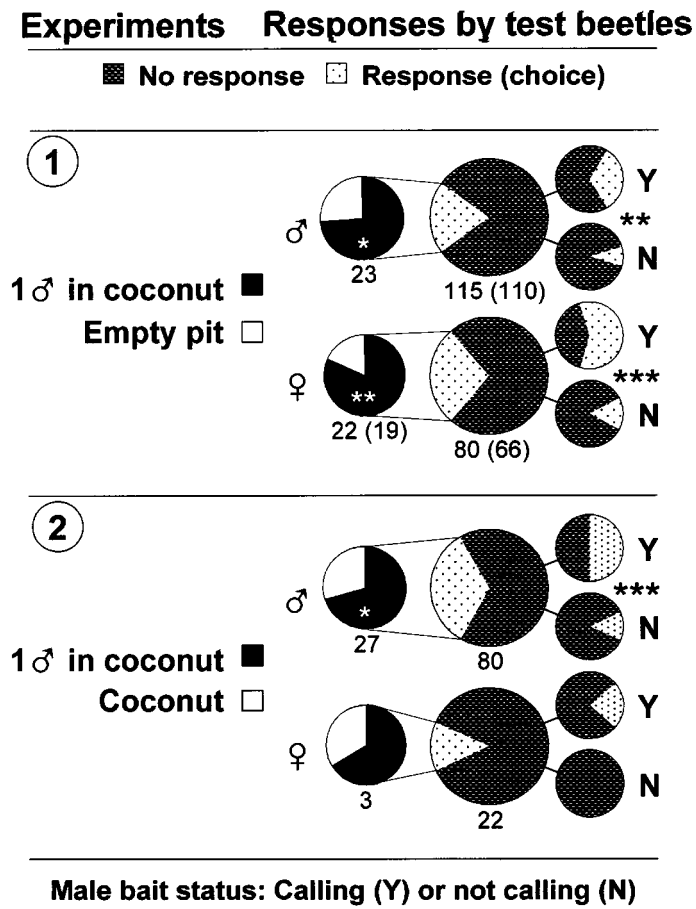


FIG. 1. Responses of *Scapanes a. australis* in five experiments (1–5) carried out in a two-choice pitfall olfactometer after 30 min. (CCRI Steward Research Station, Madang, PNG; April 1997–April 1998). Values under the pie charts (left to right) indicate the numbers of responses, of replicates and of individuals used (in parentheses). In experiments 1 and 2, the proportions of responding beetles to calling and to noncalling bait males are significantly different with $^{**}P < 0.01$ and $^{***}P < 0.001$ (χ^2 tests on contingency tables). The number of responses to the treatment is significantly greater than to the control at: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (one-tailed binomial test).

A special restraining device held the insect vertically. Recording was carried out on antennae in situ. The three antenna lamellae were separated and a glass electrode was placed on the central lamella, while the reference electrode was pushed into the eye. The antenna was flushed with a permanent moistened airstream (400 ml/min).

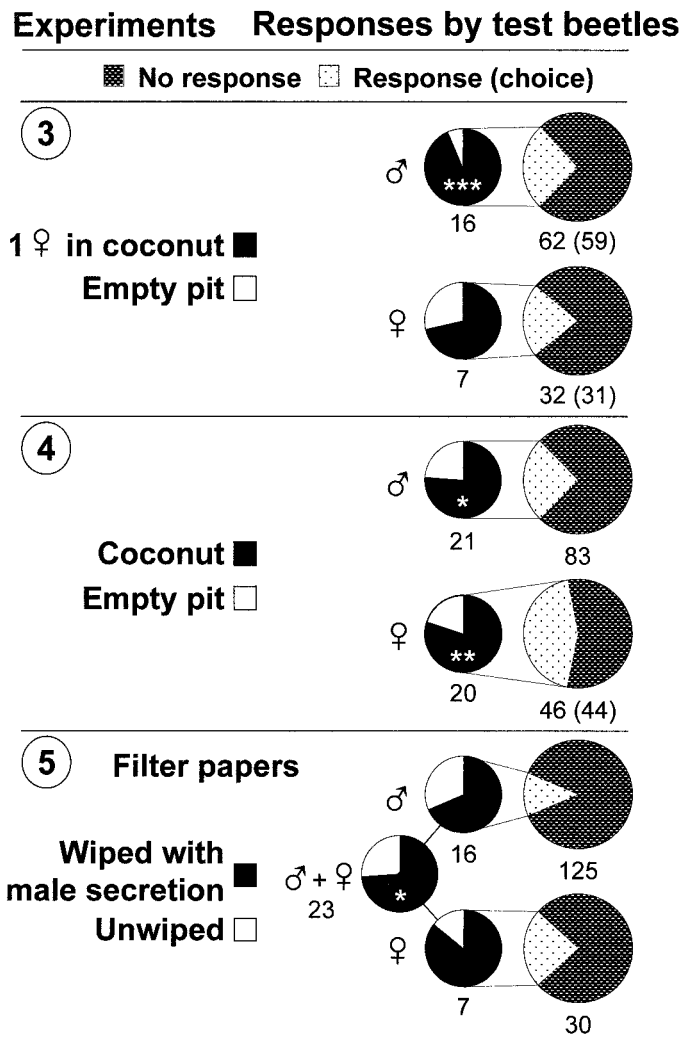


FIG. 1. Continued.

Three experiments were carried out. Experiment 6 compared the activities of various solvent extracts obtained from the effluvia collections and the wipings of abdomens on antennae from four males and four females. Experiment 7 established the responses to increasing doses of male secretion. Experiment 8 determined the responses to increasing doses (0.1, 1, 10, 100, and 1000 μg) of **1–3**, characteristic of the male secretion. The compounds were presented as 1-μl appropriate hexane solutions (hexane as control). Male secretion (10 μl) was used for comparison. As

it is an aqueous (natural) solution, a water control was also included. Experiments 7 and 8 were done only on female antennae ($N = 5$) due to a lack of males. Aliquots of the stimuli to be studied were deposited on filter papers inserted in Pasteur pipets just prior to the stimulation. The antennal responses were expressed as percentages of the amplitude measured in response to 10 μg *cis*-3-hexenol (relative EAGs) used as a reference (hexane solution). The samples were tested at a rate of 1 μl, representing 0.1 insect/wipe (wipings) and 0.1 insect/hr (effluvia). The male secretion was tested at doses of 0.1 (1 μl of a 10% solution in distilled water), 1, and 10 μl.

Statistical Analyses. The relative EAG responses were used for the calculations (STATITCF, 1991). In experiment 6, the responses to the male and female extracts were compared with the responses to the corresponding controls within each sex by Student's *t* tests. Data were processed by a one-way ANOVAs followed by a Newman-Keuls test at 5% (experiment 7) or by Dunnett's tests at 5% (experiment 8; solvent as control). The responses to the male secretion and to the water control were compared by Student's *t* tests for paired data.

Field Trapping Experiments

Semiochemical Dispensers. The characteristics of the semiochemical dispensers are listed in Table 2. They were stored at -10°C and weighed (mg precision) prior to, and at the end of, each replicate or experiment to determine the actual daily release rates of the chemicals. Details of the experiments are presented in Tables 1 and 2. All experiments were done in ENB and targeted *S. a. grossepunctatus*, except for experiments 10 and 14, done on the PNG mainland and targeting *S. a. australis*. The traps (standard) consisted of 18-liter plastic buckets with two large vents (10 $\times$ 15 cm) in the sides, with an inner plastic container (17 cm diam. $\times$ 19 cm high) perforated and fixed to the lid of the bucket (Kakul et al., 1999). All treatments (except as indicated) included three to four split sugarcane pieces. Sugarcane and one live male settled in a large sugarcane stalk when used were placed in the inner container and the semiochemical dispensers were hung outside. Traps were suspended 1 m from the ground on wood poles, or shading *Gliricidia sepium* (Jacq.), or cocoa trees after optimization (Kakul et al., 1999). Soapy water was poured into the bottom of the bucket to stop beetles from escaping. No insecticide was used. The traps were spaced 50 m apart except in experiments 10 and 14 (spaced 15 m apart). Two blocks were 100 m distant at minimum. Sugarcane was renewed and the treatments were rerandomized at each replicate (Table 1).

Chronology and Experimental Choices. To fit with the large amounts (up to 50 μl/hr) of male secretion emitted on calling, which was initially assumed to be pure pheromone, we first sought to achieve high diffusion ($\approx$1 g/day i.e., $\approx$40 mg/hr) of the synthetic **1:2:3** mixture, which was obtained with open flasks

TABLE 1. GENERAL CHARACTERISTICS OF NINE FIELD EXPERIMENTS TO TRAP *Scapanes australis* USING SYNTHETIC SEMIOCHEMICALS IN PAPUA NEW GUINEA FROM AUGUST 1998 TO FEBRUARY 2000

Experiment	Place	Date	Treatments compared[a]	Experimental design	Checks	Variable analyzed[b]
9	CCRI Tavilo Research Station, ENB (TRS)[c]	Aug 25–Sep 12, 1998	1 male + SC, SC, pheromone, pheromone + SC	8 blocks 3 replicates of 6 days	Daily 18:00–21:00	Cap./trap/replicate 2-way ANOVA
10	Malas, Madang Province, PNG Mainland[d]	Sep 21–Oct 8, 1998	1 male + SC, SC, pheromone, pheromone + SC	Randomized, 8 traps per treatment 3 replicates of 4 days	Daily	Σ cap. all traps/ replicate 1-way ANOVA
11	TRS	Oct 20–Nov 6, 1998	1 male + SC vs. 3 pheromone formulations + SC	8 blocks 3 replicates of 5 days	Daily	Cap./trap/replicate 2-way ANOVA
12	TRS	Feb 20–Mar 19, 1999	1 male + SC vs. 7 pheromone mixtures (all combinations of compounds **1**, **2** and **3**) + SC	5 blocks 4 replicates of 7 days	Daily	Cap./trap/replicate 2-way ANOVA
13	TRS	Feb 13–Mar 12, 1999	4 pheromone doses	12 blocks 4 replicates of 7 days	Daily	Cap./trap/replicate 2-way ANOVA
14	Malas	Feb 15–May 31, 1999	2 types of trap: with vs. without inner container	Randomized, 8 traps per treatment 15 replicates of 7 days		Σ cap. whole exp./trap Student's t test
15	TRS and Tokiala plantation	Jul 27–Sep 14, 1999	4 pheromone doses	12 blocks 7 replicates of 7 days	Daily	Σ cap. whole exp./trap 1-way ANOVA
16	Sikut, ENB	Oct 21–Nov 18, 1999	4 pheromone doses	12 blocks 4 replicates of 7 days	Weekly	Cap./trap/replicate 2-way ANOVA
17	Sikut	Feb. 7–28, 2000	Pheromone alone vs. 5 combinations of pheromone + a synergist (SC, Co, A, B, C)	Randomized, 6 traps per treatment 3 replicates of 7 days	Every 2–3 days	Cap./trap/replicate 2-way ANOVA

[a] Pheromone means a lure based on synthetic compounds **1** (2-butanol), **2** (3-hydroxy-2-butanone), or **3** (2,3-butanediol), identified in the secretion emitted by calling *Scapanes australis* males (Rochat et al., 2000a). See Table 2 for composition of pheromone lures. SC : Sugarcane. Co : coconut. A, B and C are synthetic mixtures based on ethanol plus ethyl acetate (at least 70% of the total and in a ca. 50:50 v/v ratio) plus various minor compounds identified as volatiles from palm and sugarcane decaying tissues (Rochat et al., 2000b).

[b] (Σ) cap.: (Sum of) captures; exp.: experiment.

[c] Mixed cocoa and coconut industrial plantation.

[d] Mixed coconut and betelnut (*Areca catechu L.*) palm family plantation.

TABLE 2. COMPOSITION, FORMULATION AND DOSES OF SYNTHETIC SEMIOCHEMICALS USED IN FIELD-TRAPPING EXPERIMENTS TO TRAP *Scapanes australis* IN PAPUA NEW GUINEA FROM AUGUST 1998 TO FEBRUARY 2000[a]

Experiment	Pheromone mixtures[b]	Dispenser types[c]	Release rates (mg/day)[d]	
			Target	Actual
9	**1:2:3** (90:5:5)	Open 50-ml HDPE flask (Kartell, Italy) with a 1100-mm^2 meniscus	>1000	2500 ± 700 (32)
10	**1:2:3** (90:5:5)	Open 50-ml HDPE flask (Kartell, Italy) with a 1100-mm^2 meniscus	>1000	2600
11	**1:2:3** (90:5:5)	30 × 25 mm (small) sachet[e]	10	8 ± 1 (24)
		90 × 95 mm reference sachet (RS)[e]	100	61 ± 5 (24)
		HDPE flasks with a reduced meniscus using a 1-cm ID tube	<200	103 ± 44 (24)
12	**1:2:3** (90:5:5)	RS	60	52 ± 8 (18)
	1:2 (90:5)	RS	54	53 ± 9 (20)
	1:3 (90:5)	RS	53	75 ± 11 (20)
	1	RS	59	71 ± 12 (19)
	2	Specific PE sachet	6	16 ± 4 (20)
	3	Specific PE sachet	1	unknown[f]
	2:3 (5:5)	Specific PE sachet	7	3 ± 2 (20)
13	**1:2:3** (90:5:5)	Specific PE sachet[g]	5	2.4 ± 1.5 (48)
		Specific PE sachet[g]	15	10 ± 1 (47)
		1 RS[g]	50	52 ± 5 (42)
		3 RSs[g]	150	165 ± 26 (41)
14	**1:2:3** (90:5:5)	1 RS[g]	50	48 ± 15 (11)
15	**1:2** (90:5)	1 RS[g]	50	53 ± 15 (12)
		2 RSs[g]	100	107 ± 10 (11)
		3 RSs[g]	150	162 ± 19 (12)
		4 RSs[g]	200	207 ± 19 (11)
16	**1:2** (90:5)	1 RS[g]	50	57 ± 4 (9)
		2 RSs[g]	100	112 ± 5 (11)
		3 RSs[g]	150	171 ± 16 (11)
		4 RSs[g]	200	223 ± 10 (9)
17	**1:2** (90:5)	Pheromone: 1 RS[g]	50	67 ± 7 (30)
		A-C mixtures: Specific plastic sachet[h]	250	325 ± 90 (36)

[a] See Table 1 for detail about the experiments.
[b] **1**, **2**, and **3** are (±)-2-butanol, 3-hydroxy-2-butanone and (±)-2,3-butanediol, respectively. Relative amounts in the mixtures given in brackets.
[c] (HD)PE: (high density) polyethylene.
[d] Mean ± standard deviation (number).
[e] Sachets made of 0.1-mm PE film ref. 50157, Bioblock Scientific, France.
[f] Undetermined due to hygroscopy of **3**.
[g] Prepared by AgriSense-BCS Ltd. (Pontypridd, United Kingdom).
[h] AgriSense-BCS Ltd. technology.

(experiments 9 and 10). To avoid loss and contamination of the chemicals, the flasks were placed in the field only during the 3 hr covering the activity periods of the species (Prior et al., 2000). Captures were recorded only at that time. As the male secretion later proved not to be pure pheromone (Rochat et al., 2000a and unpublished), the suitability of heat-sealed polyethylene (PE) sachets to release the **1:2:3** mixture at lower doses was tested (experiment 11). The role played by each of compounds **1–3** was studied in experiment 12. The **1:2:3** mixture formulated in RS (Table 2) was kept as a reference, and all possible combinations of **1–3** were prepared by removing one or two components. Lures without **1** were formulated in PE sachets, whose characteristics were adjusted in the laboratory to obtain the desired diffusion rates (Table 2). Dose–capture effect was studied extensively with the **1:2:3** (experiment 13) and the **1:2** mixtures (experiments 15 and 16), both formulated in RS. In experiment 14, we studied the effect of removing the inner plastic containers from standard bucket traps. Finally, the effect of a coattractant with the pheromone was investigated (experiment 17). Two plant material (coconut bulb and sugarcane; ca. 250 g) and the synthetic chemical mixtures A–C, known as palm odor mimics in the palm weevil, *Rhynchophorus palmarum* (L.), (Rochat et al., 2000b and unpublished) were compared for their ability to increase *S. australis* response to the pheromone.

Statistical Analysis. Most data were processed by two-way ANOVAs (F1: bait; F2: replicate) on $\mathrm{Ln}(x + 1)$ transformed data (Table 1) (STATITCF, 1991). The means were compared by Newman-Keuls tests at 5%. Due to very low captures, elementary data were pooled in experiments 10, 14, and 16 and processed similarly or with a Student's t test.

Long-Term Mass Trapping Effect on S. australis grossepunctatus Population

This was estimated by recording the captures of beetles in 14 traps installed at the border of a 40 ha area at CCRI Tavilo Research Station (TRS) during 125 weeks (April 3, 1998–August 24, 2000). The traps were the standard devices previously described. The baits were one live male settled in a sugarcane piece during the first 60 weeks, then two synthetic pheromone dispensers (RS) filled with **1:2:3** mixture and releasing about 100 mg/day. The male baits were checked every one to two weeks, replaced when dead, and the sugarcane renewed. Pheromone dispensers were replaced when necessary.

RESULTS

Olfactometer Experiments. Regardless of the type of experiment or the sex tested, ca. 75% of the beetles did not respond in the olfactometer tests, exhibiting weak or no locomotor activity. Considering the beetles that responded

(Figure 1), both sexes responded to coconut without insect (male: $P = 0.013$; female: $P = 0.006$; experiment 4) and to one male in coconut (male: $P = 0.017$; female: $P = 0.002$; experiment 1) versus blank controls. Males, but not females, responded to one female in coconut ($P < 0.001$; experiment 3) versus a blank control and to one male in coconut ($P = 0.026$; experiment 2) versus coconut. In the two experiments where one male was used as bait, the proportions of responses (whatever the choice) were greater when the bait male adopted a calling behavior than when it did not (one male in coconut versus blank: male: $\chi^2 = 9.56$, 1 *df*, $P < 0.01$; female: $\chi^2 = 15.19$, 1 *df*, $P < 0.001$; one male in coconut versus coconut: male: $\chi^2 = 11.55$, 1 *df*, $P < 0.001$; experiments 1 and 2), except for females tested with one male in coconut versus coconut with a limited number of replicates ($\chi^2 = 0.84$, 1 *df*, $P > 0.05$; experiment 2). A nonsignificant trend for a preference for the filter papers wiped with male secretion versus the unwiped filter papers was observed for both sexes (male: $P = 0.10$; female: $P = 0.14$). The effect of the male secretion proved to be significant when analyzing the responses by both sexes pooled ($P = 0.017$; experiment 5).

Electroantennography. The wiping and effluvia extracts of male origin always triggered relative EAGs of greater amplitudes than the corresponding controls. The differences were significant in most cases (Figure 2). The EAG responses elicited

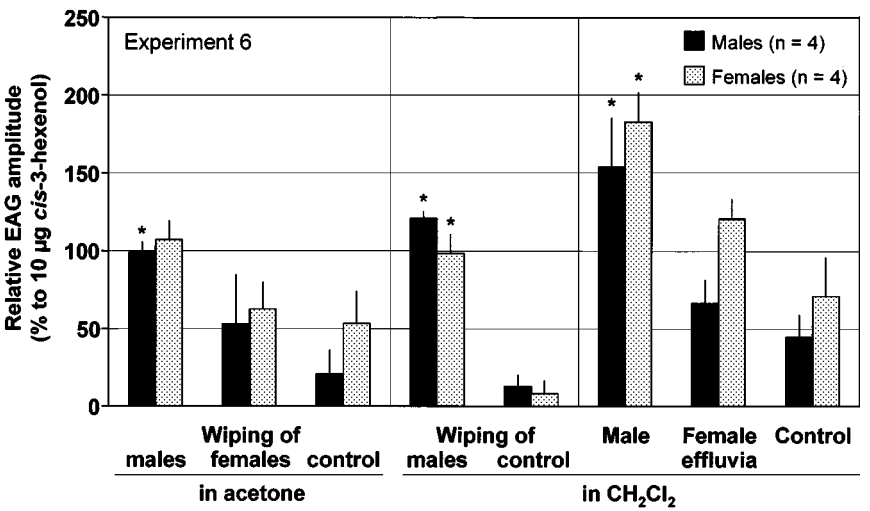


FIG. 2. Mean relative (% response to 10 μg *cis*-3-hexenol) electroantennogram responses of male and female *S. australis grossepunctatus* to wipings of male secretion (0.1 insect/wipe) and male effluvia (0.1 insect/hr) in 1-μl samples with respect to wipings and effluvia of females and corresponding solvent controls (experiment 6). *The differences with the corresponding controls are significant (Student's *t* tests at 5%).

by the extracts of female origin were not significantly different from the control. The profiles of responses by male and female antennae were similar (Figure 2). The male secretion released a log-linear dependent EAG response on the female antenna [$F(1,13) = 25.2$, $P < 0.001$; $r^2 = 0.66$], each dose releasing a significantly greater EAG than the lower one. The responses were greater than to pure air (control: 0.19 ± 0.04) at any doses. The mean relative EAG to 10 μl male secretion (0.94 ± 0.05) was of the same level as those recorded to extracts from wipings of males at 0.1 insect/wipe doses (1.07 ± 0.12 and 0.98 ± 0.12). A weak dose–response effect was recorded to acetoin: $F(5,20) = 5.52$, $P < 0.01$, and to 2,3-butanediol: $F(5,20) = 3.95$, $P < 0.05$, in the range tested (0.1 to 1000 μg on filter paper; Figure 3). Only the relative EAGs recorded to the highest dose tested were significantly greater than to hexane. The mean relative EAGs to every dose of acetoin and 2,3-butanediol were lower than to 1 μl male secretion. A more marked dose effect was recorded to 2-butanol: $F(5,20) = 57.38$, $P < 0.001$ (Figure 3). Relative EAGs to the 100 and 1000 μg doses were significantly greater than to hexane. One microliter of male secretion elicited a relative EAG intermediary with

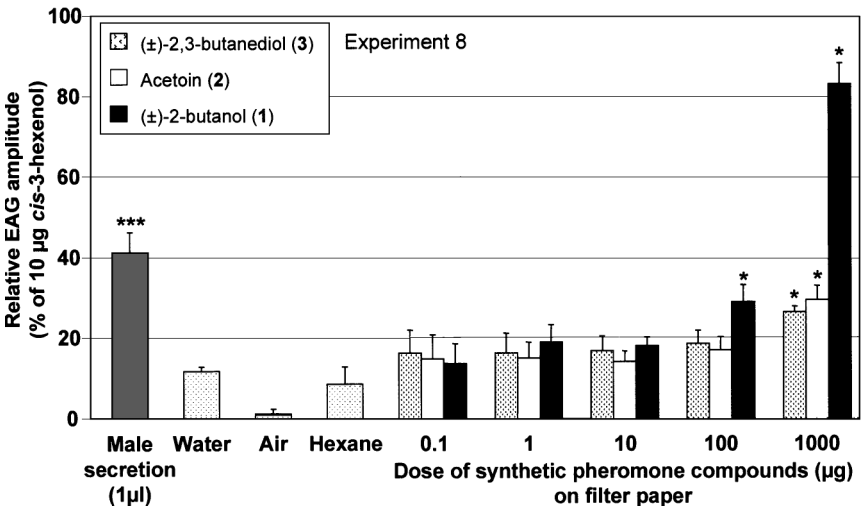


FIG. 3. Mean relative (% response to 10 μg *cis*-3-hexenol) electroantennogram responses of female *Scapanes australis grossepunctatus* ($N = 5$) to increasing doses of the synthetic components identified in the male secretion: **1**: (±)-2-butanol, **2**: acetoin, and **3**: (±)-2,3-butanediol (mixtures of stereo-isomers) diluted in hexane relative to 1 μl male secretion and controls (experiment 8). Doses are amounts put on a filter paper in 1 μl solution. Lines associated with bars represent the standard errors of the means. Synthetic chemicals: bars topped by one asterisk indicate responses different from the control (hexane; Dunnett's test at 5% after one-way ANOVAs). Male secretion is compared to distilled water by a Student's *t* test for paired data. ***Difference significant at $P < 0.001$.

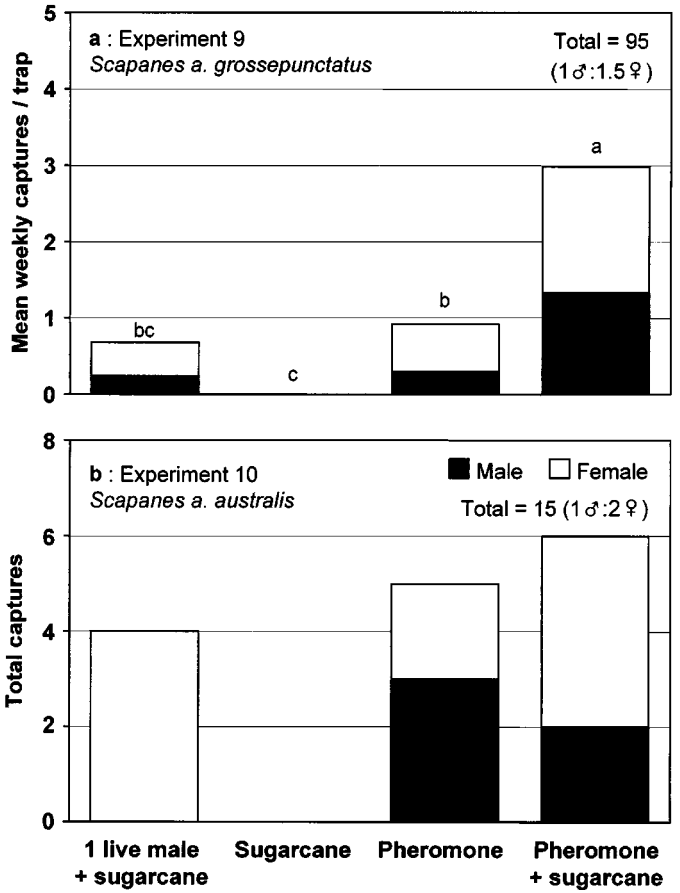


FIG. 4. Responses of *Scapanes australis* to traps baited with sugarcane, synthetic pheromone [90:5:5 v/v mixture of (±)-2-butanol, acetoin, and (±)-2,3-butanediol emitted at ca. 2.5 g/day from open polyethylene flasks], or both compared to traps baited with one live male in sugarcane. (a) Responses by *S. a. grossepunctatus* in CCRI Tavilo Research Station (experiment 9); (b) responses by *S. a. australis* in Malas (experiment 10). Treatments associated with same letters do not differ in effect based on ANOVAs followed by Newman-Keuls test at 5%. See Tables 1 and 2 for other data about experiments.

100 and 1000 μg 2-butanol, lower than in previous experiments but greater than the water control ($t = 6.74$, 4 *df*, two-tailed test, $P < 0.01$).

Field-Trapping Experiments. The actual release rates of the semiochemical dispensers used are detailed in Table 2. In experiment 9 (Figure 4a), the bait and replicate effects were significant: $F(3,77) = 22.02$, $P < 0.001$ and

$F(2,77) = 4.13$, $P < 0.05$, respectively. Both factors interacted: $F(6,77) = 4.41$, $P < 0.001$, the captures by the second replicate were twice as abundant as the others. **1:2:3** + SC caught more beetles (4×) than only SC (no capture) and male + SC control. Captures by only **1:2:3** were different from zero. Captures by only **1:2:3** and male + SC control were equivalent. The **1:2:3** mixture was emitted at a mean dose of 2500 mg/day. Captures in experiment 10 followed the same pattern as in experiment 9 (Figure 4b) with a similar pheromone release, but they were weak and no significant effect of the bait could be disclosed: $F(3,31) = 1,40$, $P > 0.2$.

In experiment 11 (Figure 5), the bait and replicate effects were significant: $F(3,77) = 13.95$ and $F(2,77) = 18.49$, respectively, $P < 0.001$. Both factors interacted, $F(6,77) = 4.56$, $P < 0.001$, due to contrasted levels of captures during the three replicates. **1:2:3** + SC either released by open flasks (103 mg/day) or the RS (61 mg/day) caught as many beetles and more (2×) than SC + **1:2:3** released

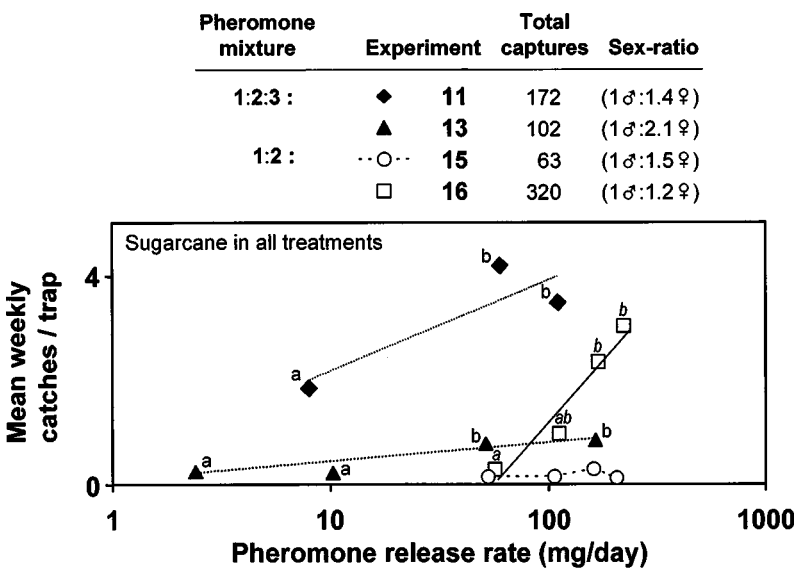


FIG. 5. Responses of *Scapanes australis grossepunctatus* to traps baited with various doses of ternary [**1:2:3**: (±)-2-butanol, acetoin, and (±)-2,3-butanediol in 90:5:5 v/v ratio] or binary [**1:2:** (±)-2-butanol and acetoin in 90:5 v/v ratio] synthetic pheromone mixtures plus sugarcane pieces during four experiments in East New Britain (PNG). The pheromone was formulated in heat-sealed polyethylene sachets except for the highest dose in experiment 11, a modified open polyethylene flask. See Table 2 for actual release rates and detail about dispensers. Experimental characteristics are given in Table 1. Within each experiment, doses associated with same letters led to equivalent captures based on an ANOVA followed by Newman-Keuls test at the 5% level.

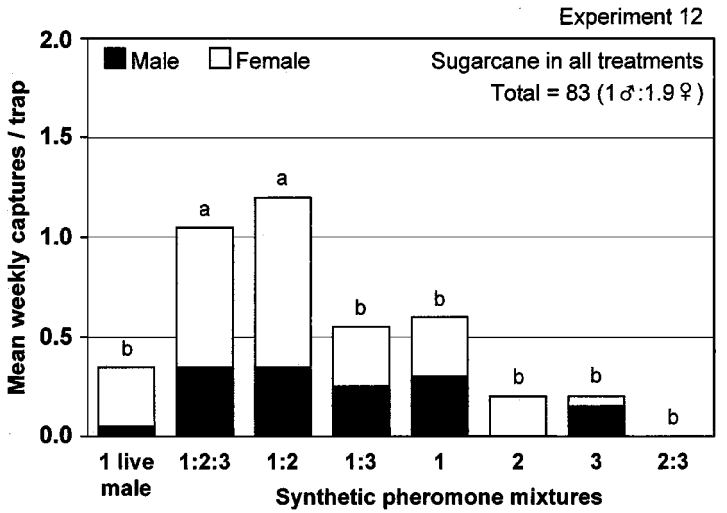


FIG. 6. Responses of *Scapanes australis grossepunctatus* to traps baited with various mixtures of synthetic (±)-2-butanol **(1)**, acetoin **(2)**, and (±)-2,3-butanol **(3)** obtained by removing one or two components from the complete mixture in 90:5:5 (v/v) ratio with respect to traps baited with one live male in sugarcane [experiment 12: CCRI Tavilo Research Station (TRS)]. Pheromone lures were formulated in heat-sealed polyethylene sachets (Table 2). Treatments associated with same letters do not differ in effect based on an ANOVA followed by Newman-Keuls test at 5%. See Table 1 for other data.

by the small sachet (8 mg/day). All treatments with **1**:**2**:**3** caught more beetles than the male + SC control. Release by the PE sachets was much less variable than by the open flasks.

In experiment 12 (Figure 6), the bait effect was significant: $F(7,124) = 7.34$, $P < 0.001$. Absence of **1** in the synthetic baits resulted in very low captures. The combinations of **1:2** + SC and **1:2:3** + SC caught more (2×) beetles than all other baits, including the male + SC control. The latter group had statistically equivalent effects. Addition of **3** to either **1** or **1:2** did not increase the captures. Baits with **1** emitted mean pheromone doses ranging from 53 to 71 mg/day.

In experiment 14, only 71 beetles (1 male: 1.4 females) were caught during 105 days in 16 traps. The captures were greater in traps with than without (0.43 vs 0.16 beetle/trap/week) the inner plastic boxes ($t = 2.63$, 12 *df*, $P = 0.011$, one-tailed test). The pheromone was emitted at 48 mg/day.

In the dose–response experiments (Figure 5), the dose effect was significant: $F(3,165) = 5.75$, $P < 0.001$ in experiment 13 (**1:2:3** mixture). The two lower doses (2.4 and 10 mg/day) had equivalent effects, with smaller captures than the two higher doses (52 and 165 mg/day). A trend for a log-linear relationship was

observed between the captures and the pheromone dose released. With **1:2** mixture, no effect of the dose (207, 162, 107, and 53 mg/day on an average) was recorded: $F(3,33) = 0.82$, $P > 0.5$ during experiment 15; but a dose effect was observed in experiment 16: $F(3,165) = 7.92$, $P < 0.001$. The two higher doses (223 and 171 mg/day) had equivalent effects, with greater catches than the lowest one (57 mg/day). The fourth dose (112 mg/day) had an intermediary effect. A log-linear relationship was observed between the mean weekly captures per trap and the mean daily pheromone release: $F(1,2) = 129.7$, $P < 0.01$; $r^2 = 0.98$. Eighty percent of the beetles were captured during the second week (significant replicate effect: $F(3,165) = 44.17$, $P < 0.001$).

In experiment 17 (Figure 7), the coattractant added to the pheromone (67 mg/day) had a significant effect on the captures: $F(5,85) = 15.92$, $P < 0.001$. Coconut allowed significantly greater captures (70% total) than any other bait (6×). The other baits added to the pheromone did not improve the captures obtained with the pheromone alone, especially the synthetic mixtures A–C, emitted at a mean dose of 325 mg/day.

Long Term Mass Trapping. A total of 2173 beetles were removed from the treated area during 125 weeks in the 14 traps monitored (Figure 8). The weekly

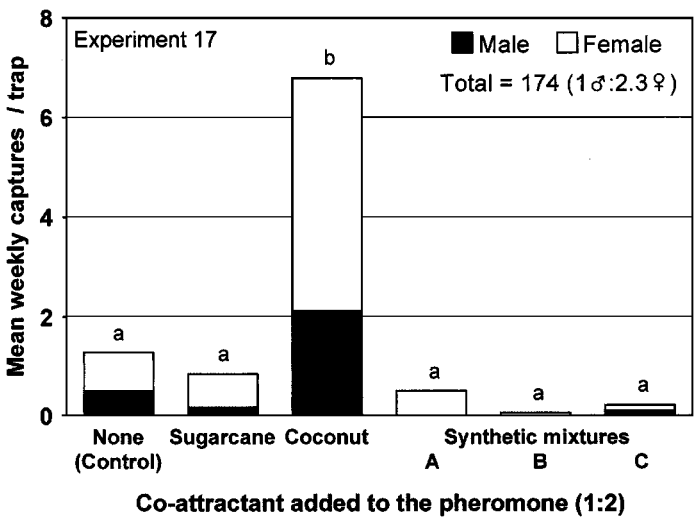


FIG. 7. Coattractant effect of coconut and sugarcane pieces and of three synthetic chemical mixtures (A–C) known for pherosynergistic properties in palm weevils (*Rhynchophorus* spp.) compared to synthetic *Scapanes australis* pheromone [**1:2:** 90:5 v/v mixture of (±)-2-butanol and acetoin] emitted at 67 mg/day (experiment 7, Sikut, East New Britain). Treatments associated with same letters do not differ in effect based on an ANOVA followed by Newman-Keuls test at 5%.

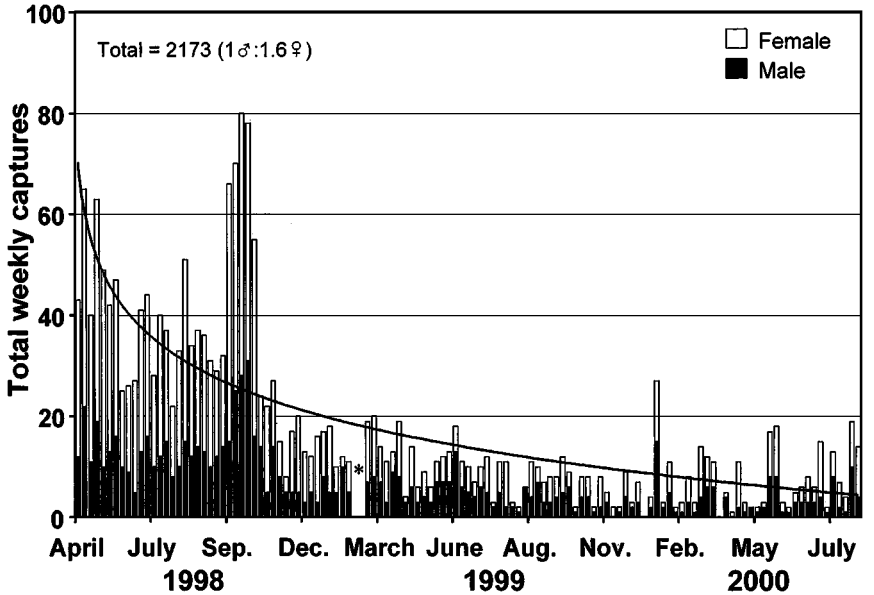


FIG. 8. Weekly captures of *Scapanes australis grossepunctatus* by 14 traps baited with either one live male or two dispensers of synthetic pheromone [**1:2:3:** 90:5:5 v/v mixture of (±)-2-butanol, acetoin, and (±)-2,3-butanediol emitted at about 100 mg/day] plus sugarcane pieces in a 40-ha area of cocoa-coconut plantings in CCRI Tavilo Research Station during continuous monitoring from April 3, 1998, to August 24, 2000. The curve is a log-linear fitting of the total captures versus the time from the beginning. *Indicates no trapping during 2 weeks.

captures dropped 30 weeks after the installation of the monitoring system, from 43 ± 15 (mean $\pm$ SD) in the initial 30 weeks to 14 ± 5 beetles in the next 30 weeks. The captures followed a log-linear relationship with time along the period of monitoring: $F(1,121) = 150.81$, $P < 0.001$, $r^2 = 0.55$. A marked peak of captures that could not be correlated with a change in the baits put in the traps was observed in October 1998.

DISCUSSION

Field trapping with synthetic compounds unambiguously demonstrated that 2-butanol and acetoin are key components of the male pheromone of *S. australis grossepunctatus*, acting for long-range attraction on both sexes. It established the basis for the development of the pheromone-based mass trapping as a new control method against this pest.

The two-choice pitfall olfactometer gave the first clue of an attraction of both sexes to males placed on coconut pieces, mainly to calling males, suggesting the emission of a volatile pheromone. However, the experiments were not conclusive, especially due to the high proportion of beetles that did not respond. Definite evidence for attraction to calling males was obtained a few months later by field trapping (Prior et al., 2000). The response to the male secretion was also weak and again not conclusive. The use of a suboptimal dose might have contributed to that result since the pheromone was diluted in a solvent that was itself as volatile as its major component. As the beetles did not respond to the **1:2:3** reference mixture in further olfactometer experiments (unreported), our olfactometer device and test procedure might have not been appropriate to the species, although good responses to pheromones were recorded in the same device from two other large palm beetles: *O. rhinoceros*, another Dynast (Saïd et al., unpublished data), and the weevil, *R. palmarum* (Rochat et al., 1991). In contrast to the two latter species, *S. australis* has developed a complex behavior sequence associated with sex communication and selection (Prior et al., 2000), the optimal realization of which was probably not possible in our laboratory conditions.

Electroantennography, especially coupled with gas chromatography, has been used as an efficient tool to disclose the pheromones of most scarab species studied to date (review in Leal, 1998), as well as *Oryctes* spp. (Gries et al., 1994; Hallet et al., 1995; Morin et al., 1996). *S. australis* appears to be an exception. Indeed, both the natural and the synthetic male pheromone compounds elicited EAGs of weak amplitudes with a low signal-to-noise ratio. The significant EAGs from the natural pheromone samples supported the existence of a male pheromone, but attempts to concentrate the extracts led to a loss of the EAG activity. This was logical, due to the high volatility of 2-butanol, the major pheromone component, undetermined at that time. GC-EAG recordings from the extracts did not reveal consistent EAGs from any compound (unreported). EAGs from the synthetic pheromone components greater than the control could only be recorded at very high doses (1 mg/filter paper), likely not relevant in natural situations. The existence of highly sensitive olfactory neuroreceptors to pheromones has been described in several scarab beetles [e.g., *Anomala cuprea* Hope (Leal and Mochizuki, 1993), *O. rhinoceros* (Morin et al., 1996; Renou et al., 1998), and *Phyllopertha diversa* Waterhouse (Hansson et al., 1999; Nikonov et al., 2001)]. The low threshold of response to the synthetic pheromone measured in field trapping suggests the existence of such receptors in *S. australis*, but electroantennography did not reveal them, and single sensillum recordings would be necessary for confirmation.

In field trapping experiments, *S. a. australis* individuals were captured by the synthetic pheromone designed from the analyses of *S. a grossepunctatus* male secretion (experiments 10 and 14). However, captures were limited and did not seem to reflect the beetle abundance in the experimental plots, as estimated by the

number found in palm galleries (Beaudoin-Ollivier, unpublished). This and the lack of responses to the **1:2:3** mixture in the olfactometer may reflect a difference in *S. a. australis* male pheromone composition with respect to *S. a. grossepunctatus* that could not be verified. As *S. a. australis* is not a pest in the PNG mainland, investigations were not pursued to clarify that point, and the following refers to *S. a. grossepunctatus*.

The synthetic mixture of 2-butanol, acetoin, and 2,3-butanediol, which reproduced the specific bouquet emitted by the male secretion, attracted beetles from both sexes. The combination of 2-butanol and acetoin was necessary and sufficient to obtain captures equivalent to those by the ternary mixture. The two compounds are actual pheromone components with long-range activity. In contrast, 2,3-butanediol seemed not to be involved in long-range attraction. It may play a role in short-range recognition or be involved in the specificity of the pheromone message.

As initially reported, beetles always responded to the racemic chemicals and stereoisomer mixtures used. This agreed with the composition of the natural pheromone: a mixture of enantiomers and stereoisomers (Rochat et al., 2000a). The specificity of the pheromone message remains puzzling since it relies on the use of short-chain chemicals, that are not optically pure. While acetoin and 2,3-butanediol are commonly produced by microorganisms, 2-butanol appears to be a less common molecule in nature (Robinson et al., 1999). The specificity of the message could rely on the large amount of chemical emitted (160 μg $\mathbf{1}+\mathbf{2}+\mathbf{3}$ on average) (Rochat et al., 2000a and unpublished) during the brief period of calling (1 hr) (Prior et al., 2000) associated with a specific adaptation of the olfactory apparatus, although the latter was not evidenced by electroantennography. Beetles were captured by 2.4- to 2600-mg/day doses. No sign of inhibition was detected at the higher doses. The capture at given doses depended on the experiment and likely on the population level in and around the experimental plots. Differences in the dose–capture relationships were not correlated with the presence or absence of 2,3-butanediol, which confirmed the lack of long range activity of this compound.

More females (average: 60%) than males were caught in the synthetic pheromone than in the male-baited traps in all experiments. The proportion was the same during the 125-week mass-trapping campaign. This sex ratio is opposite to that of the beetles found in the galleries in coconut palms (Prior et al., 2000). Females were considered rarer and more mobile than males. The use of synthetic pheromone clearly contradicted the first assessment and confirmed the second. The male pheromone is an aggregation one according to the classical definition (Nordlund and Lewis, 1976). However, calling males had a marked territorial behavior and got rid of all males in their vicinity by fighting (Prior et al., 2000). Individuals were scattered but certainly not aggregated on trees. Males were mostly alone or with one, rarely two, females. Groups comprising more than two males

on the same palm were rare, and males were then settled in separate galleries (Prior et al., 2000). Considering these ecological elements, the male pheromone was probably originally a sex pheromone. Males might have evolved to respond to their own sex signal to gain possession of a suitable mating and feeding site from fighting rivals.

Although captures from the only synthetic pheromone were observed, the presence of plant material enhanced trap catches. As earlier experiments with only plant controls led to no catch at all, the effect is likely synergistic. In a comparative experiment, coconut, a natural host plant of the beetle, appeared more efficient than sugarcane. As using coconut material was not possible on a large scale, sugarcane, much cheaper and easier to handle, was used in most experiments. It was accepted by the live males, and the level of captures obtained over 2.5 years with sugarcane appears to be acceptable from a pest control perspective. In contrast, the synthetic chemical mixtures that exhibited interesting pherosynergistic properties in certain palm weevils were not active on *S. australis*. Either the dose used was not optimal or the beetle does not use the same chemical signature to recognize its host plants. The results may, nevertheless, constitute a basis for further investigations aimed at identifying plant pherosynergists active in *S. australis*.

The 125-week campaign carried out in the TRS plantation indicated that mass trapping can severely depress the local *S. australis* population. The pheromone-based mass trapping provides success for controlling females, which is of special importance in any pest control program and particularly in the present case since the breeding sites where they spend the most time are difficult to access (Beaudoin-Ollivier et al., 1999). It is also targeting the males that are primarily damaging the palms. Our various experiments also reveal that outbreaks of adults occur during the year (intense peaks of catches in experiments 11 and 16 and in the long-term monitoring), for which detection and rapid elimination are important to improve the control of this pest. The trapping components (traps and attractants) have been optimized (Kakul et al., 1999 and unpublished). Despite rather large active doses, the synthetic pheromone is cheap, and its formulation is easy. Great hopes are riding on a decisive breakthrough in controlling *S. australis* after the severe coconut recession that has occurred PNG in recent years. The optimal trap layouts and the impact of the method on the damage level in coconut plots are currently being investigated as is the development of simultaneous trapping of both *S. australis* and BPW, for which a pheromone is already available (Oehlschlager et al., 1995).

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SEMIOCHEMICALS OF THE SCARABAEINAE: VI. IDENTIFICATION OF EAD-ACTIVE CONSTITUENTS OF ABDOMINAL SECRETION OF MALE DUNG BEETLE, *Kheper nigroaeneus*

B. V. BURGER* and W. G. B. PETERSEN

Laboratory for Ecological Chemistry
Department of Chemistry
University of Stellenbosch
Stellenbosch 7600, South Africa

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Abstract—Using gas chromatography with flame ionization detection (FID) and electroantennographic detection (EAD) in parallel, and employing chiral and achiral capillary columns, three constituents of the abdominal sex-attracting secretion of male *Kheper nigroaeneus* dung beetles were found to elicit reproducible EAD responses in male and female *K. nigroaeneus* antennae. One of these constituents is present in the secretion in such a small quantity that it could not be detected by FID, and it was not identified. The other constituents were identified as 3-methylindole (skatole) and (*R*)-(+)-3-methylheptanoic acid.

Key Words—Scarabaeinae, *Kheper nigroaeneus*, dung beetle, sex attractant, sex pheromone, insect semiochemicals, EAD, electroantennographic detection.

INTRODUCTION

The coprophagous fauna associated with the mammals of Africa plays an important role in the recycling of nutrients and in the destruction of the habitat of many dung breeding flies (Heinrich and Bartholomew, 1979). During the summer months when dung beetle activity reaches its peak, hardly any flies are found in game reserves with normal herbivore populations. In contrast, areas a few kilometers outside these reserves, where the cultivation of land or other farming practices have resulted in a decrease in the dung beetle population, the indigenous people are often plagued by swarms of flies. Similar problems are experienced in Australia,

* To whom correspondence should be addressed. E-mail: lecus@maties.sun.ac.za

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where cattle were introduced without the associated insect fauna, resulting in the deterioration of pastures (Waterhouse, 1974) and the uncontrolled increase of the fly population (Hughes, 1970).

Based on their nesting behavior, dung beetles of the subfamily Scarabaeinae can be divided into three groups (Halffter and Matthews, 1966; Bornemissza, 1969). The paracoprids construct their nests under the dung pad by excavating tunnels in which dung is packed, the endocoprids excavate a chamber in the dung pad itself, forming brood-balls within this chamber, and the telecoprids detach a portion of dung from the pad, rolling it some distance from the dung source before burying it. The majority of species of dung beetles found in southern Africa are crepuscular paracoprids (87%), although the diurnal telecoprids are also numerous, even if represented by fewer species (12%) (Ferreira, 1972). The telecoprid genus *Kheper* is confined mainly to the hot Lowveld areas of Natal and the Transvaal, extending into tropical Africa.

Telecoprid behavior is considered to have evolved from competition for dung, as dung is the meeting place of the sexes and is vital for mating success. Dung beetles, are attracted to dung and arrive by the hundreds at fresh dung where they either immediately start feeding or burrow into the dung. Within a few minutes, they distinguish between the sexes, determine the state of maturity of the opposite sex, and enlist the cooperation of the selected partner in molding and rolling a brood-ball. If the male has been unsuccessful in finding a female in or on the dung pad, he might fly away and start secreting a sex attractant or start constructing a dung ball on his own. The male *K. nigroaeneus* first buries a brood-ball before assuming the typical secretion-producing stance at the entrance to the burrow in which the ball has been buried, or even when he is not in possession of a dung ball (Edwards and Aschenborn, 1988). Supporting his body on the front two pairs of legs, the male raises his abdomen and lowers his head so that his body is at a relatively steep angle. Repeated rapid extension of the hind legs results in the extrusion of a white proteinaceous material from tiny openings in a depression on either side of the first abdominal sternite. The secretion is supplied from a glandular complex underlying the depressions. The movement of the hind legs results in the secretion being broken up into tiny particles between brushes on the tibia and comblike structures on the abdomen of the male. Small puffs of secretion are, thus, released into the air. Amputation of one of the hind legs of a beetle results in thin-strand fibers resembling cotton wool being formed on that side of its abdomen. The tibial brushes are rudimentary in the females. When the presence of an attracted female is detected by a calling male, he challenges the female by raising his forelegs and advancing on her. Her reaction is a submissive lowering of the forelegs, followed by antennal contact and acceptance by the male. If the male has not already prepared and, in the case of *K. nigroaeneus*, buried a dung ball, the male detaches a portion of dung from the pad and proceeds to roll both the dung and the female clinging to it and compacting the dung, away from the dung pad.

Once the dung ball is buried, copulation occurs below the soil, the male returning to the surface after a few days, while the female remains below to lay an egg and to brood the developing larva (Tribe, 1975, 1976).

The abdominal secretions produced by *Kheper* species consist of a visible proteinaceous material that acts as a pheromone-disseminating carrier material for minute quantities of a complex mixture of volatile organic compounds. A comparison of the secretions of *K. lamarcki*, *K. nigroaeneus*, and *K. subaeneus* revealed that similar, but not identical, proteins with molecular masses of ca. 15 kDa are the major constituents of the proteinaceous fractions of each of these secretions (Burger et al., 1990). The major volatile constituents of the secretion of *K. lamarcki* were found to be hexadecanoic acid, 2,6-dimethyl-5-heptenoic acid, and (*E*)-nerolidol (Burger et al., 1983). The volatile fractions of the secretions of these *Kheper* species are, in fact, complex mixtures, each containing at least 150 constituents such as, among others, hydrocarbons, straight- and branched-chain fatty acids and methyl and ethyl esters of these, and other fatty acids. The volatile compounds present in the secretions of these three species are similar, but only a few are common to two or all three of the species. The secretions of these insects contain many chiral long-chain compounds. Because the enantiomers of these compounds cannot be separated with the current technology, it was decided to suspend attempts to identify all the volatile constituents of the secretions in favor of the identification of the EAD-active constituents. In this paper, identification of two EAD-active constituents of the abdominal secretion of male *K. nigroaeneus* is presented.

METHODS AND MATERIALS

Collection and Purification of Carrier Material. The pentane, dichloromethane, and chloroform used for extraction purposes were of residue analysis grade (Merck). All Pyrex glassware used in the handling of the material was heated to 500°C in an annealing oven to remove any traces of organic material. Spatulas, syringes, etc., used in handling the secretion, were cleaned by rinsing with the dichloromethane specified above.

Adult *K. nigroaeneus* dung beetles were trapped in Mkuzi Game Reserve in Kwazulu Natal, South Africa, during the early summer months by baiting pitfall traps (Tribe, 1976) with fresh horse dung. On arrival in Stellenbosch, one hind leg was removed from all male beetles. A small percentage of the beetles died within a week or two after removal of a leg. This was, however, the best method to prevent dispersal of the secretion that, if not brushed off, collects at one side of the abdomen. The male and female beetles were kept at subtropical temperatures in greenhouses, the floors of which were covered with about 15 cm of moist sandy soil. Permits were granted for the collection and removal from the game reserve of 150 males and a small number of females annually for the duration of this project.

When disturbed, male dung beetles immediately stop secreting the attractant to investigate, and they may even disappear underground together with a female. Males observed to assume the attractant secreting posture were, therefore, fenced off from other beetles with aluminum strips. In some cases, the production of secretion continued for up to 2 hr. Without touching the body of the secreting male, the secreted material was removed periodically from its incapacitated side with ophthalmic forceps to prevent contamination with dust particles and the evaporation of the pheromone from the carrier material. In the meantime, the vial with material already collected was kept cool in an icebox. Tiny dust and dung particles picked up from the abdomen of the insects by the emerging secretion were carefully removed from the secretion under a microscope. Only about 5–10% of the males produced secretion in captivity, and in most instances, secretion production lasted only a short while and yielded too little material for gas chromatographic analysis of the secretion from individual insects. Small samples, therefore, had to be pooled for analysis. However, when secretion production was kept up for longer periods, it was possible to analyze the secretion from individuals. In a typical sample preparation, 3 mg of the secretion was sonicated for 1 min with 50 μl of chloroform in a Reacti-Vial, after which the suspension was centrifuged at 3000 rpm for 10 min. The white carrier material was concentrated in the upper layers of the solvent, while the remaining dust particles were precipitated. The solvent, containing soluble organic material, was carefully removed from between these two layers of solid material with a 100-μl syringe. The process was repeated a number of times, and extracts were pooled and concentrated for analysis of the volatile components of the secretion.

Analytical Methods. Gas chromatographic determinations were carried out with a Carlo Erba 4160 gas chromatograph with parallel flame ionization and electroantennographic detection (FID/EAD). The instrument was equipped with a 40 m $\times$ 0.3 mm glass capillary column coated with OV-1701-OH at a film thickness of 0.4 μm. Helium was used as carrier gas at a linear velocity of 28.5 cm/sec at 40°C, and a temperature program of 2°C/min from 40°C to 250°C was employed. Chiral gas chromatographic analyses were done on a glass column (25 m $\times$ 0.3 mm) coated with 10% heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin in OV-1701-OH at a film thickness of 0.25 μm, using H_2 as carrier gas at a linear velocity of 50 cm/sec. The instrumentation for gas chromatographic analysis of dung beetle secretions with FID and EAD recording in parallel, with the use of dung beetle antennae as sensing elements has been described by Burger and Petersen (1991). In short, an antenna was inserted lengthwise into the air duct, as this arrangement produced a less noisy baseline than positioning the antenna sideways in the airstream, a position in which the club of the antenna is subjected to the effect of the turbulent mixing of the effluent and ambient air. The antenna was protected against heat radiation from the gas chromatograph by a heat shield cooled with water at 18°C. Electrical responses in the antenna were recorded via Ag–AgCl electrodes placed in

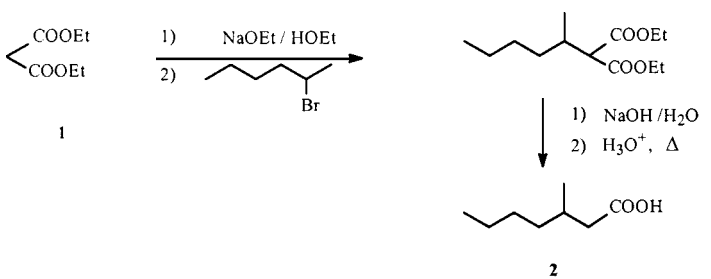
pipets filled with saline solution containing NaCl (7.5 g/liter), $CaCl_2$ (0.21 g/liter), KCl (0.35 g/liter), and $NaHCO_3$ (0.2 g/liter), as well as polyvinylpyrrolidone K90 (Fluka, 49 g/liter) to increase its viscosity (Van der Pers, 1980). Antennal responses were amplified with a Murphy Developments AMS-025 amplifier and were recorded on an Omniscribe recorder.

Mass spectra were recorded on a Carlo Erba QMD 1000 quadrupole mass spectrometer with the column and gas chromatographic conditions specified above. ^{1}H and ^{13}C NMR spectra of synthetic compounds and intermediates were recorded at 299.905 MHz and 75.42 MHz, respectively, at 25°C on a Varian VXR 300 NMR spectrometer.

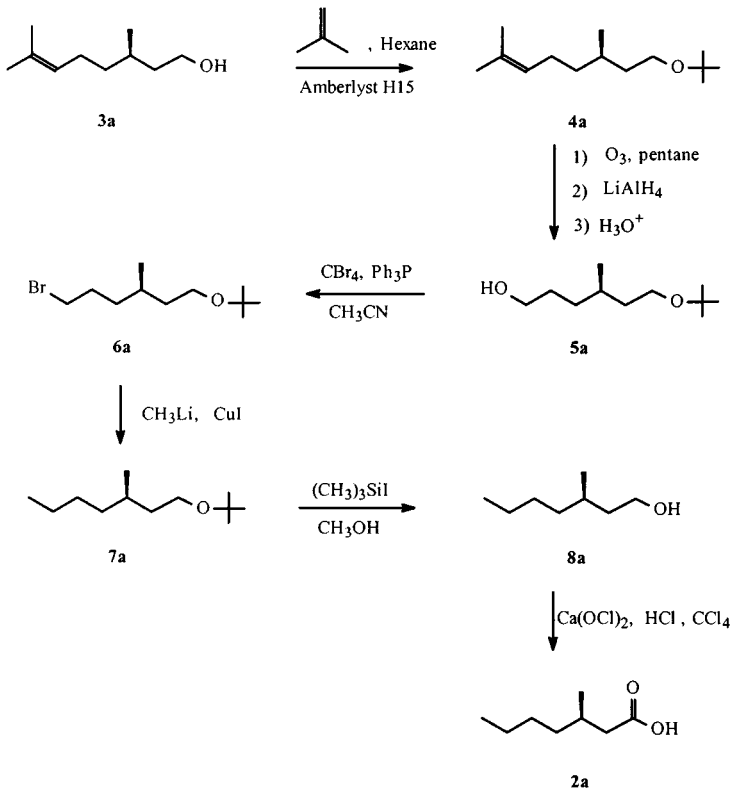
3-Methylheptanoic acid was isolated from extracts of the abdominal secretion by preparative gas chromatography on a glass column (40 m × 0.6 mm) coated with the apolar phase PS-255 at a film thickness of 1.0 μm. A 1:10 effluent splitter was used, and fractions were collected in $CDCl_3$ for NMR analysis. ^{1}H NMR ($CDCl_3$): $\delta = 2.357$ (dd, 1H, C**H**H-2, $^2J_{2a,2b} = 15.0$ Hz, $^3J_{2a,3} = 5.9$ Hz); 2.153 (dd,1H, CH**H**-2, $^2J_{2b,2a} = 15.0$ Hz, $^3J_{2b,3} = 8.0$ Hz); 1.96 (m, 1H, C**H**-3); 1.45–1.15 (m, 6H, C$\mathbf{H}_2$-4,5,6); 0.894 (t, 3H, C$\mathbf{H}_3$-7, $^3J_{7,6} = 6{,}7$ Hz); 0.970 (d, 3H, C$\mathbf{H}_3$-8, $^3J_{8,3} = 6.6$ Hz).

Reference Compounds. 3-Methylindole and starting compounds were purchased from Sigma-Aldrich (Cape Town, South Africa).

Racemic 3-methylheptanoic acid (**2**) was synthesized from diethyl malonate according to Scheme 1. Diethyl malonate (**1**) (23.22 g; 145 mmol) was deprotonated with a solution of sodium ethoxide prepared from sodium (3.22 g; 140 mmol) and absolute ethanol (100 ml), and then alkylated with 2-bromohexane (23.11 g; 140 mmol) (Vogel, 1978). The resulting diethyl ester was saponified by refluxing it with a solution of potassium hydroxide (20 g) in water (30 ml), after which the reaction mixture was acidified and refluxed until the evolution of carbon dioxide had ceased. The conventional isolation procedures gave *rac*-3-methylheptanoic acid (**2**) as a colorless oil (12.94 g; 64%); ^{1}H NMR ($CDCl_3$): $\delta = 2.356$ (dd, 1H, C**H**H-2, $^2J_{2a,2b} = 14.9$ Hz, $^3J_{2a,3} = 6.0$ Hz); 2.145 (dd, 1H, CH**H**-2, $^2J_{2b,2a} = 14.9$ Hz, $^3J_{2b,3} = 8.1$ Hz); 1.96 (m, 1H, C**H**-3); 1.44–1.14 (m, 6H,



SCHEME 1. Synthesis of racemic 3-heptadecanoic acid.



3a **4a** **6a** **5a** **7a** **8a** **2a**

SCHEME 2. Synthesis of (*R*)-(+)-3-methylheptanoic acid.

C**H**$_2$-4,5,6); 0.894 (t, 3H, C**H**$_3$-7, $^3J_{7,6} = 6.6$ Hz); 0.969 (d, 3H, C**H**$_3$-8, $^3J_{8,3} = 6.6$ Hz).

(*R*)-(+)-*3-Methylheptanoic acid* (**2a**) was synthesized from (*R*)-(+)-3,7-dimethyl-6-octen-1-ol [(*R*)-(+)-β-citronellol] according to Scheme 2. Isobutene was slowly introduced into a suspension of Amberlyst H-15 (2.88 g) in a solution of (*R*)-(+)-3,7-dimethyl-6-octen-1-ol (**3a**) [9.91 g; 63.5 mmol, 98% pure, $[\alpha]_D^{19}$ + 5.3° (neat)] in hexane (100 ml) at room temperature for 12 hr (Alexakis et al., 1988). Removal of the catalyst and solvent gave a colorless oil (13.47 g) containing 99.9% (GC-MS) of (*R*)-(+)-8-*tert*-butoxy-2,6-dimethyl-2-octene (**4a**); $[\alpha]_D^{25}$ + 3.26° (neat). MS: *m*/*z*(%) = 156(6), 138(4), 137(5), 123(6), 109(7), 95(28), 81(67), 69(97), 57(100), 55(48), 43(35), 41(94). ^{1}H NMR ($CDCl_3$): $\delta = 1.599$ (d, 3H, C**H**$_3$-1, $^4J_{1,3} = 1.4$ Hz); 5.102 (m, 1H, =C**H**-3, $^3J_{3,4} = 7.1$ Hz, $^4J_{1,3} = {}^4J_{3,9} =$ 1.4 Hz); 1.980 (m, 2H, C**H**$_2$-4); 1.46–1.21 (m, 2H, C**H**$_2$-5); 1.62–1.47 (m, 1H, C**H**-6); 1.62–1.47 (m, 1H, C**H**H-7); 1.46–1.21 (m, 1H, CH**H**-7); 3.33 (m, 1H,

C**H**H-8); 3.38 (m, 1H, CH**H**-8); 1.679 (d, 3H, C**H**$_3$-9, $^4J_{9,3} = 1.4$ Hz); 0.890 (d, 3H, C**H**$_3$-10, $^3J_{10,6} = 6.5$ Hz); 1.185 (s, 9H, (C**H**$_3$)$_3$). ^{13}C NMR ($CDCl_3$): $\delta = 25.72$ (q, C-1), 131.01 (s, C-2), 124.98 (d, C-3), 25.51 (t, C-4), 37.30 (t, C-5), 29.66 (d, C-6), 37.77 (t, C-7), 59.80 (t, C-8), 17.62 (q, **C**H$_3$-9), 19.72 (q, **C**H$_3$-10), 72.51 (s, C-1′), 27.62 (q, 3**C**H$_3$).

Ozone was passed through a solution of the unsaturated ether (**4a**) (13.42 g; 63.3 mmol) in pentane (100 ml) until a sharp color change from colorless to blue was observed (Marvel and Nichols, 1941). Reduction of the ozonide with lithium aluminum hydride at –10°C, the usual work-up, and column chromatography on silica gel gave (*R*)-(+)-6-*tert*-butoxy-4-methyl-1-hexanol (**5a**) (7.02 g, 59%) in 99.2% purity (GC-MS); $[\alpha]_D^{25} + 1.95°$ (neat). MS: *m*/*z*(%) = 173(2), 132(6), 131(15), 115(28), 97(49), 69(40), 59(38), 57(100), 55(78), 43(23), 41(55). ^{1}H NMR ($CDCl_3$); $\delta = 3.627$ (t, 2H, OC**H**$_2$-1, $^3J_{1,2} = 6.6$ Hz); 1.72–1.47 (m, 2H, C**H**$_2$-2); 1.47–1.26 (m, 2H, C**H**$_2$-3); 1.72–1.47 (m, 1H, C**H**-4); 1.72–1.47 (m, 1H, C**H**H-5); 1.47–1.26 (m, 1H, CH**H**-5); 3.382 (qd, 1H, C**H**H-6, $^2J_{6a,6b} = 14.7$ Hz, $^3J_{6a,5a} = 8.6$ Hz, $^3J_{6a,5b} = 6.7$ Hz); 3.360 (qd, 1H, CH**H**-6, $^2J_{6b,6a} = 14.6$ Hz, $^3J_{6b,5a} = 8.6$ Hz, $^3J_{6b,5b} = 7.0$ Hz); 0.938 (d, 3H, C**H**$_3$-7); 1.185 [s, 9H, (C**H**$_3$)$_3$]. ^{13}C NMR ($CDCl_3$): $\delta = 63.37$ (t, C-1), 30.20 (t, C-2), 33.05 (t, C-3), 29.72 (d, C-4), 37.67 (t, C-5), 59.53 (t, C-6), 19.72 (q, **C**H$_3$-7), 72.52 (s, C-1′), 27.61 (q, 3**C**H$_3$).

Bromination of the alcohol (**5a**) (6.80 g; 36.20 mmol) with tetrabromomethane (14.37 g; 43.33 mmol) and triphenylphosphine (10.42 g; 39.72 mmol;) in acetonitrile (40 ml) (Christol et al., 1978; Slagle et al., 1981) gave (*R*)-(+)-1-bromo-6-*tert*-butoxy-4-methylhexane (**6a**) as a colorless oil (8.74 g, 96.4%) in 86% purity (GC-MS); $[\alpha]_D^{25} + 3.46°$ (neat). MS: *m*/*z* (%) = 237(3), 235(3), 179(9), 177(10), 150(2), 148(2), 137(9), 135(10), 123(6), 121(7), 109(6), 107(7), 97(92), 69(85), 59(88), 57(100), 56(77), 55(92), 43(86), 41(95). ^{1}H NMR ($CDCl_3$): $\delta = 3.354$ (t, 2H, C**H**$_2$-1, $^3J_{1,2} = 7.0$ Hz); 1.98–1.76 (m, 2H, C**H**$_2$-2); 1.49–1.21 (m, 2H, C**H**$_2$-3); 1.72–1.42 (m, 1H, C**H**-4); 1.69–1.50 (m, 2H, C**H**$_2$-5); 3.396 (qd, 1H, C**H**H-6, $^2J_{6a,6b} = 14.3$ Hz, $^3J_{6a,5a} = 8.9$ Hz, $^3J_{6a,5b} = 6.9$ Hz); 3.354 (qd, 1H, CH**H**-6, $^2J_{6b,6a} = 14.9$ Hz, $^3J_{6b,5a} = 8.8$ Hz, $^3J_{6b,5b} = 6.9$ Hz); 0.900 (d, 3H, C**H**$_3$-7, $^3J_{7,4} = 6.6$ Hz); 1.85 [s, 9H, (C**H**$_3$)$_3$]. ^{13}C NMR ($CDCl_3$): $\delta = 34.29$ (t, C-1), 30,41 (t, C-2), 35.57 (t, C-3), 29.35 (d, C-4), 37.87 (t, C-5), 59.53 (t, C-6), 19.72 (q, **C**H$_3$-7), 72.49 (s, C-1′), 27.62 (q, 3**C**H$_3$).

Treatment of the bromide (**6a**) (7.35 g; 29.3 mmol) with lithium dimethylcuprate in ether at 0°C (Johnson and Dutra, 1973) and isolation of the product in the usual manner gave (*R*)-1-*tert*-butoxy-3-methylheptane (**7a**) as a colorless oil (4.04 g, 74%) in 85% purity (GC-MS). MS: *m*/*z* (%) = 171(2), 113(3), 84(3), 71(28), 69(7), 59(25), 57(100), 56(18), 55(17), 43(35), 41(43). ^{1}H NMR ($CDCl_3$): $\delta = 3.370$ (qd, 1H, C**H**H-1, $^2J_{1a,1b} = 14.9$ Hz, $^3J_{1a,2a} = 8.8$ Hz, $^3J_{1a,2b} = 6.0$ Hz); 3.344 (qd, 1H, CH**H**-1, $^2J_{1b,1a} = 14.9$ Hz, $^3J_{1b,2a} = 8.7$ Hz, $^3J_{1b,2b} = 6.5$ Hz); 1.62–1.46 (m, 2H, C**H**$_2$-2); 1.70–1.46 (m, 2H, C**H**-3); 1.46–1.05 (m, 6H, C**H**$_2$-4,5,6); 0.899 (t, 3H, C**H**$_3$-7, $^3J_{7,6} = 6.6$ Hz); 0.871 (d, 3H, C**H**$_3$-8,

$^3J_{8,3} = 6.6$ Hz); 1.185 [s, 9 H, (C**H**$_3$)$_3$]. ^{13}C NMR ($CDCl_3$): $\delta = 59.88$ (t, C-1), 37.87 (t, C-2), 25.65 (d, C-3), 36.88 (t, C-4), 29.21 (t, C-5), 23.01 (t, C-6), 14.17 (q, C-7), 19.78 (q, **C**H$_3$-8), 72.42 (s, C-1′), 27. 63 (q, 3**C**H$_3$).

Treatment of the *tert*-butoxy ether (**7a**) (3.21 g; 17.2 mmol) with iodotrimethylsilane (6.18 g; 30.91 mmol) in dichloromethane (50 ml) (Jung and Lyster, 1977) gave (*R*)-3-methyl-1-heptanol (**8a**) (2.24 g) in quantitative yield and 86% purity (GC-MS). MS: *m*/*z*(%) = 112(2), 97(3), 84(52), 83(25), 70(77), 69(37), 57(38), 56(67), 55(98), 43(100), 44(38), 41(87). ^{1}H NMR ($CDCl_3$): $\delta = 3.654$ (t,2H, OC**H**$_2$-1, $^3J_{1,2} = 6.6$ Hz); 1.66–1.48 (m,2H, C**H**$_2$-2); 1.69–1.48 (m,1H, C**H**-3); 1.47–1.11 (m,6H, C**H**$_2$-4,5,6); 0.896 (t, 3H, C**H**$_3$-7, $^3J_{7,6} = 6.7$ Hz); 0.894 (d, 3H, C**H**$_3$-8). ^{13}C NMR ($CDCl_3$): $\delta = 61.28$ (t, C-1), 40.01 (t, C-2), 27.35 (d, C-3), 36.82 (t, C-4), 29.20 (t, C-5), 22. 97 (t, C-6), 14.12 (q, C-7), 19.66 (q, **C**H$_3$-8).

Oxidation of the alcohol (**8a**) with various reagents such as chromic acid and permanganate gave the acid (**2a**) in very poor yields. The best results were obtained with calcium hypochlorite as oxidizing agent (Kabalka et al., 1990). Concentrated hydrochloric acid (7 ml) was, thus, added dropwise to a stirred suspension of hypochlorite (8.8 g; 61.5 mmol) in water (40 ml) giving a bright yellow solution. A mixture of *tert*-butanol (30 ml) and tetrachloromethane (20 ml) was added, followed by the addition of (*R*)-3-methyl-1-heptanol (**8a**) (1.87 g; 14.4 mmol) in *tert*-butanol (10 ml) over a period of 4 min. The reaction mixture was stirred for 5 hr, and the organic material extracted exhaustively with tetrachloromethane. The combined extracts were washed with a solution of sodium metabisulfite until it was colorless. The organic acid was extracted with sodium carbonate, and this basic solution was extracted thoroughly with ether to remove all non-acidic products. The water layer was carefully acidified with hydrochloric acid and the organic acid extracted with ether. The usual work-up procedures gave (*R*)-(+)-3-methylheptanoic acid (**2a**) as a colorless oil (1.14 g, 55%) in 99.3% purity (GC-MS); $[\alpha]_D^{25} + 4.94^\circ$ (neat); 96.2% ee by gas chromatography on heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin. MS: *m*/*z* (%) = 144(3), 129(8), 115(3), 101(7), 87(55), 85(28), 84(33), 73(20), 69(22), 61(26), 60(100), 56(20), 55(38), 45(27), 43(75), 42(26), 41(65). ^{1}H NMR ($CDCl_3$): $\delta =$ 2.355 (dd,1H, C**H**H-2, $^2J_{2a,2b} = 14.9$ Hz, $^3J_{2a,3} = 5.9$ Hz); 2.144 (dd, 1H, CH**H**-2, $^2J_{2b,2a,} = 14.9$ Hz, $^3J_{2b,3} = 8.1$ Hz); 1.96 (m, 1H, C**H**-3); 1.46–1.11 (m, 6H, C**H**$_2$-4,5,6); 0.894 (t, 3H, C**H**$_3$-7, $^3J_{7,6} = 6.7$ Hz); 0.968 (d, 3H, C**H**$_3$-8, $^3J_{8,3} = 6.6$ Hz). ^{13}C NMR ($CDCl_3$): $\delta = 190.40$ (s, C-1), 41.56 (t, C-2), 30.16 (d, C-3), 36.37 (t, C-4), 29.11 (t, C-5), 22.78 (t, C-6), 14.07 (q, C-7), 19.72 (q, **C**H$_3$-8).

(*S*)-(−)-*3-methylheptanoic acid* was synthesized in an analogous manner from (*S*)-(−)-β-citronellol in 51% yield, 99.2% purity (GC-MS), and 96.8% ee; MS: *m*/*z*(%) = 144(3), 129(9), 115(10), 101(7), 87(55), 85(38), 84(33), 73(20), 69(21), 61(25), 60(100), 56(19), 55(36), 45(26), 43(73), 42(25), 41(65), 39(30).

RESULTS AND DISCUSSION

A typical FID gas chromatogram of an extract of the abdominal secretion of the male *K. nigroaeneus* is shown in Figure 1. A faster programming rate and the injection of more material were found to produce a better signal-to-noise ratio in the EAD recording. Dung beetle antennae survived in EAD experiments for more than 12 hours. Figure 2 shows the first part of a gas chromatographic analysis with EAD and FID recording in parallel at a programming rate of 4°C/min. A female antenna was used as the EAD-sensing element in this analysis. Although male and female antennae mostly produced reproducible and qualitatively similar results, a few examples of male and female antennae responding reproducibly to a few other long-chain constituents of the secretion were encountered over a period of several years. Because these EAD analyses could not be reproduced with other antennae, identification of these compounds could not be verified by EAD analyses of the synthetic compounds. However, they were always long-chain fatty acids such as hexadecanoic acid and/or the methyl or ethyl esters of long-chain acids. It is difficult to explain these results, and this phenomenon was not investigated in more detail.

One explanation for the presence of a large number of long-chain alkanes, alcohols, fatty acids, etc., in the abdominal secretion could be that these compounds augment the pheromone disseminating capacity of the proteinaceous carrier material. However, the fact that the antennae of the male and female dung beetles on occasion seemed to respond to some of these compounds could indicate that, depending on circumstances, they possibly have a semiochemical function.

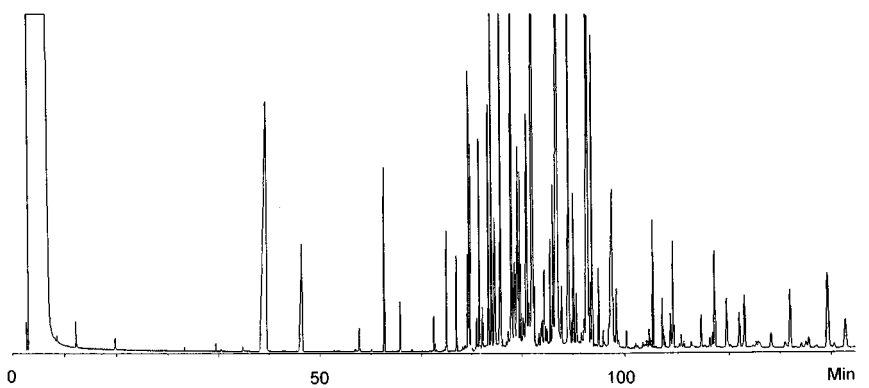


FIG. 1. Total ion chromatogram (GC-MS) of an extract of the male abdominal secretion of the dung beetle *Kheper nigroaeneus.* Gas chromatographic conditions: 40 m × 0.3 mm-ID glass column coated with OV-1701-OH at a film thickness of 0.375 μm and programmed at 2°/min from 40° to 250°C (hold); injector temperature 220°C; FID 250°C.

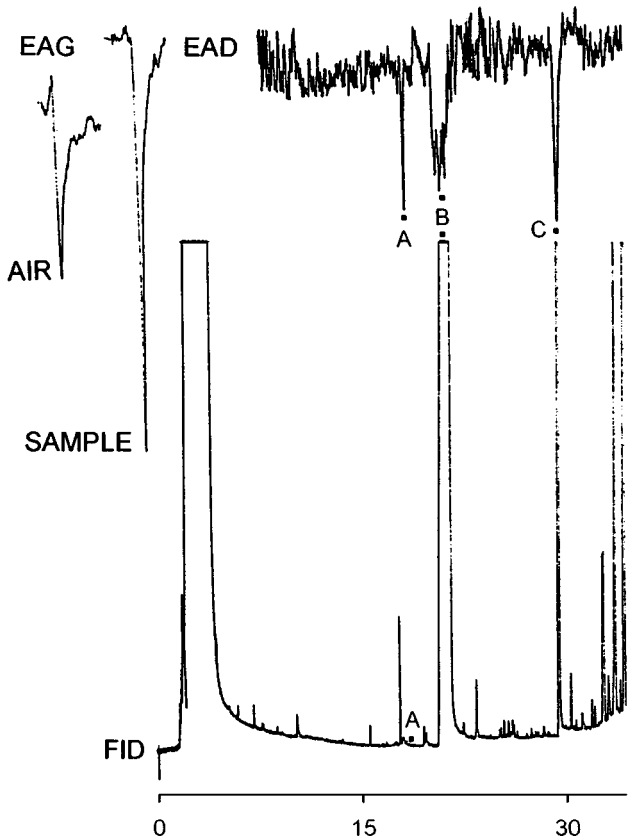


FIG. 2. First part of a gas chromatogram of an extract of the male abdominal secretion of the dung beetle *Kheper nigroaeneus* obtained by EAD/FID recording in parallel using a female antenna as detector element. Corresponding peaks in the EAD and FID chromatograms are indicated with a dot. Gas chromatographic conditions as in Figure 1 but with a programming rate of 4°C/min from 40° to 250°C (hold); EAD interface line 250°C; antenna and humidified air at 22°C. Assignment of EAD-active constituents: A = unidentified; B = (*R*)-(+)-3-methylheptanoic acid; C = skatole.

Although some of these long-chain compounds are common to the four *Kheper* species that were investigated, it is noteworthy that there were clearly large qualitative and quantitative differences in their secretions. This could be construed as evidence that the volatile constituents of the secretions are involved in the transmission of chemical signals. It is also possible, however, that the EAD and gas chromatographic techniques used in this study were not suitable for the detection of such long-chain compounds.

Constituent A, eluting at 18.55 min in the gas chromatographic analysis of the volatile organic fraction of the abdominal secretion shown in Figure 2, gave reproducible EAD responses, but was invisible in FID gas chromatograms despite relatively large samples having been used for the analysis. The low concentration in which this compound is present in the secretion and the fact that it was impossible to collect large enough quantities of abdominal secretion so far has precluded its identification.

Constituent B, eluting at 20.92 min, was initially identified as 5-methylheptanoic acid. However, it was found that the retention time and mass spectra of synthetic 5-methylheptanoic acid are different from those of the natural compound. 5-Methylheptanoic acid, furthermore, did not elicit any EAD response in the antennae of male or female *K. nigroaeneus*. All the available abdominal secretions and extracts of the secretion accumulated over more than eight years were combined for preparative gas chromatography, and enough material was isolated for ^{1}H NMR analysis of this acid, which was identified as 3-methylheptanoic acid.

Racemic 3-methylheptanoic acid (**2**) was synthesized from diethyl malonate (**1**) and 2-bromohexane according to Scheme 1. (*R*)-(+)-3-Methylheptanoic acid (**2a**) was synthesized from (*R*)-(+)-3,7-dimethyl-6-octen-1-ol [(*R*)-(+)-β-citronellol] (**3a**) according to Scheme 2 and was found to have mass and ^{1}H NMR spectra identical to those of the natural compound. This synthetic enantiomer was found to be EAD active. The *S* enantiomer was synthesized from (*S*)-(−)-β-citronellol according to an analogous synthetic route and was found to be EAD inactive. The two enantiomers are separable on a capillary column coated with a mixture of the stationary phase OV-1701-OH and heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin, the *S* enantiomer eluting first from this column. Finally, a gas chromatographic analysis with FID and EAD detection in parallel and using this chiral column confirmed the EAD activity of the second eluting *R* enantiomer.

The third EAD-active constituent of the secretion, C, eluting at 29.21 min in the gas chromatogram shown in Figure 2, was identified as 3-methylindole (skatole), which has also been identified as a constituent of the abdominal secretion of *K. lamarcki*. Synthetic 3-methylindole was found to be EAD active.

One of the goals of this research was the development of a pheromone-based monitoring system for some of the *Kheper* species as a possible method to evaluate certain aspects of the ecological status of areas where these dung beetles are found. So far, all attempts at devising a field test with which the constituents of the secretion could be evaluated have been unsuccessful. The primary source of attraction is dung, and in tests with constituents of the secretion of *K. lamarcki*, for example, no dung beetles were attracted to pitfall traps baited with hexadecanoic acid, 2,6-dimethyl-5-heptenoic acid, (*E*)-nerolidol, and skatole. In the case of *K. lamarcki*, initial indications that more females than males are attracted to pitfall traps baited with dung plus these compounds than to traps baited only with dung proved to be statistically insignificant. In this regard, however, it must also be taken

into consideration that the ratio of males to females constantly changes due to the fact that the females stay underground for a while after mating. The sex ratio could also be influenced by other factors, such as the localized availability of dung and differences in the texture and moisture content of the soil in different parts of the area where the tests were done.

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IDENTIFICATION OF THE SEX PHEROMONE OF *Holotrichia reynaudi*

ANDREW WARD,[1,6] CHRIS MOORE,[2,*] V. ANITHA,[3] JOHN WIGHTMAN,[4] and D. JOHN ROGERS[5]

[1]*Farming Systems Institute*
Department of Primary Industries
PO Box 23, Kingaroy, Qld 4610, Australia

[2]*Farming Systems Institute*
Department of Primary Industries
665 Fairfield Road, Yeerongpilly, Qld 4105, Australia

[3]*International Crops Research Institute for the Semi-Arid Tropics*
Patancheru, Andhra Pradesh 502 324, India

[4]*International Pest Management*
Conondale Cottage, Lot 7 Stanley River Road
Maleny Qld 4552, Australia

[5]*Farming Systems Institute*
Department of Primary Industries
Meiers Road, Indooroopilly, Qld 4068, Australia

[6]*Current address: Department of Primary Industries and Fisheries*
PO Box 1346, Katherine, NT 0851, Australia

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Abstract—The male attractant pheromone of the scarab beetle *Holotrichia reynaudi*, an agricultural pest native to southern India, was extracted from abdominal glands of females with hexane and analyzed by gas chromatography–mass spectrometry. Field testing of the candidate chemicals, indole, phenol, and anisole, both alone and as binary mixtures, led us to conclude that anisole was the major component of the sex pheromone. Neither male nor female beetles were attracted to indole or phenol on their own. Similarly, when indole and anisole were combined, the attractiveness of the solution did not increase over that obtained with anisole alone. However, combination of phenol and anisole did alter the attractiveness of anisole, with fewer male beetles attracted to the binary mixture than to anisole on its own. The behavior of female beetles was not altered by any of the chemicals tested. Anisole is also the sex pheromone of *H. consanguinea*, making this the first known example of two melolonthine scarabs sharing the same pheromone.

* To whom correspondence should be addressed. E-mail: moorec@dpi.qld.gov.au

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Key Words—Coleoptera, Scarabaeidae, Melolonthinae, *Holotrichia reynaudi*, anisole, indole, phenol, pheromone.

INTRODUCTION

In recent years, there has been a variety of studies examining the chemical ecology of white grubs injurious to agricultural crops. As a result, the sex pheromones of a number of species from the subfamilies Melolonthinae and Rutelinae have been successfully identified (Leal, 1998). These include the pheromones used by the melolonthine species *Costelytra zealandica* (Henzell and Lowe, 1970), *Holotrichia parallela* (Leal et al., 1992), and *Holotrichia consanguinea* (Leal et al., 1996).

Sex pheromones may play an important role in maintaining the reproductive isolation of closely related species. Despite this, beetles from two genera of rutelines have been found to share either the same pheromone or a complex of pheromones. Leal et al. (1994) observed that *Anomala albopilosa sakishimana* and *Anomala cuprea* shared the same pheromone complex. Similarly, Potter (1980) reported that *Cyclocephala lurida* (formerly *C. immaculata*) and *Cyclocephala borealis* appeared to share the same pheromones. In both cases, reproductive isolation was maintained by either temporal or spatial means. Although a number of rutelines have been observed sharing the same pheromones, no similar observations have been made in the melolonthines. This paper reports the first known record of the same phenomenon in the Melolonthinae.

Holotrichia is a genus of the melolonthine scarabs that occurs across the Indian subcontinent and through southeast and east Asia. A number of species, including *H. reynaudi* and *H. consanguinea*, are major agricultural pests in India. Leal et al. (1996) identified anisole as the pheromone used by *H. consanguinea*. In this paper, we report that *H. reynaudi* also uses anisole as a sex pheromone.

METHODS AND MATERIALS

Analytical Procedures. Gas chromatography–mass spectrometry (GC-MS) was carried out on a VG Trio-2000 instrument (VG Biotech, Altrincham, UK), equipped with a split/splitless injector and nonpolar DB5-MS column (30 m $\times$ 0.25 mm; 0.25 μm). The column was held at 40°C for 2 min, then raised to 260°C at 10°C/min, where it was held for 6 min.

Extracts. Abdominal glands were excised from four calling females and placed in a screw top vial containing 2 ml of solvent (hexane). The vial containing the glands was air freighted to Australia for analysis along with 2 ml of the original hexane, which acted as a blank.

Field Testing. Behavioral responses to the candidate pheromones, anisole, indole, and phenol were evaluated over two years at the International Centre for

Research in the Semi Arid Tropics (ICRISAT) near Hyderabad, in the southern Indian State of Andhra Pradesh. Indole and anisole were also tested at Mahbubnagar (approximately 100 km southwest of Hyderabad) in 2000. In 2000, field testing of anisole and indole was completed at ICRISAT over five nights, June, 7–8 and June, 12–14. In 2001, anisole and phenol were tested at ICRISAT over four nights, June, 4–7. The test site at ICRISAT, in both 2000 and 2001, was an area of approximately 1500 m^2 in which eight distinct patches of *H. reynaudi*'s host tree (*Zizyphus mauritiana*) were growing. The trees had a maximum height of approximately 2 m. Testing was carried out at Mahbubnagar over one night (June 16, 2000). The test site consisted of a U-shaped border to a commercial peanut field surrounded by another of *H. reynaudi*'s host trees, *Butea monosperma*.

At ICRISAT, in 2000, anisole, indole, anisole + indole, or a control (empty vial) was assigned to each patch of trees, giving two replicates of each treatment. Phenol was not tested until 2001, as it was not clearly evident in the initial samples (see Results and Discussion for details). The location of each chemical was rerandomized each night. The anisole, indole, and phenol were purchased as laboratory reagents in India (anisole from s.d. fine-chem pvt. Ltd., indole from Loba Chemie, Bombay; phenol from Qualigens Fine Chemicals, Glaxo India Limited) and were undiluted. Approximately 5 g of each of the candidate chemicals was placed in a 20-ml glass vial. This was approximately the quantity of anisole soaked onto sponges and placed into trees by J. N. Vijayvergia (personal communication) in on-farm beetle-management trials with *H. consanguinea* in Rajasthan. The glass vials were taped to the stem of the host tree approximately 10 min before the anticipated time of beetle emergence. In the case of the anisole + indole mixture, equal quantities of the two compounds were mixed together by weight. Each pheromone station was separated by at least 10 m. The attractiveness of each compound was assessed by counting the number of beetles attracted to each vial. The sex ratio was also recorded. A similar sampling procedure was adopted at Mahbubnagar. Beetle flights around the anisole and the anisole + indole trees during the period of attractiveness of the chemicals were too intense (>200 beetles) for accurate counts to be made. A funnel trap baited with anisole was also placed in the middle of the field at this site.

In 2001, field tests at ICRISAT compared the attractiveness of anisole, phenol, anisole + phenol and empty (clean) vials to *H. reynaudi* beetles. Beetle attraction was monitored with water traps, which consisted of plastic bowls 10 cm deep × 35 cm diam. Two 30-cm-high aluminum baffles, slotted to give a right angle fit, were attached to the top of each bowl. The glass vials, into which the test compounds were placed, were accommodated in the top center of the baffle. The bowl contained 4–5 cm of water + one drop of detergent. Data were analyzed by using ANOVA following a log $(x + 1)$ transformation. Means were separated using least significant differences.

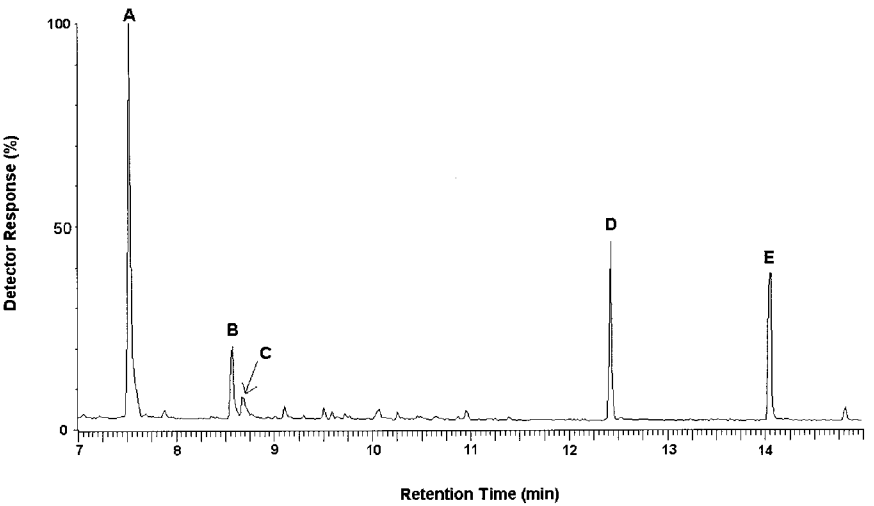


FIG. 1. GC-MS output from crude extracts taken from the abdominal glands of *H. reynaudi*. Peak A, anisole; B, dimethyltrisulfide; C, phenol; D, naphthalene; E, indole.

RESULTS AND DISCUSSION

GC-MS analysis of the crude extracts from the abdominal glands gave a clean profile (Figure 1). There were a number of relevant peaks. These were identified as indole (E), methoxybenzene (anisole, A), and phenol (C). They were the same as those identified by Leal et al. (1996) in the crude gland extracts taken from the closely related species, *H. consanguinea.* There were two additional peaks, naphthalene (D) appeared in both the solvent and the extract, suggesting that it was a contaminant. The other peak (B), was dimethyltrisulfide. As it was not possible to freight the samples in a frozen or chilled form, the gland tissues had started to decay by the time analysis was undertaken. This compound is a common component of volatiles emitted from animal wastes, biocultures, and anaerobically decomposing animal tissue (see, for example Niedziella et al., 2000). No record of the connection of this material with insect semiochemical phenomena has been found.

Early samples freighted to Australia, on which the synthetic formulation for field testing was based, had been insufficiently concentrated to reveal phenol in the original total-ion-current chromatograms. The samples had, however, been transported more carefully, and generation of mass chromatograms corresponding to phenol (*m*/*z* 94) and dimethyltrisulfide (*m*/*z* 126) from raw data acquired earlier from these samples revealed traces of phenol and the absence of dimethyltrisulfide. Although phenol has previously been identified as the pheromone used by

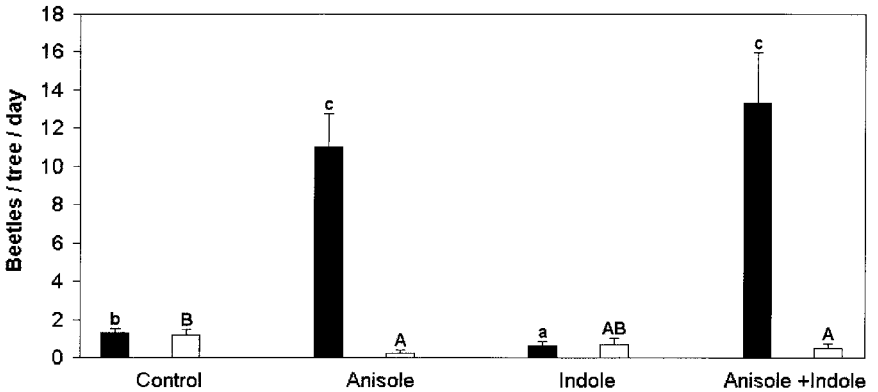


FIG. 2. Number of *H. reynaudi* beetles attracted to vials containing anisole, indole and a mixture of anisole + indole. Solid bars represent the number of male beetles and open bars represent the number of female beetles. Bars of the same color marked with the same letter are not significantly different at $P < 0.05$ using least significant differences.

Costelytra zealandica (Henzell and Lowe, 1970), field testing was, of necessity, confined to anisole and indole in 2000.

The results of the field testing at ICRISAT in 2000 demonstrated that anisole was highly attractive to male *H. reynaudi* (Figure 2). After emerging from the soil, male beetles flew towards the trees baited with vials containing anisole and the mixture of anisole + indole. More male beetles were attracted to these trees than to control trees or trees baited with indole alone [$F(3,36) = 64.97$, $P < 0.001$]. When the males reached the vials containing anisole, they flew around them and attempted to either copulate with the vials or with other male beetles. This activity commenced at about 19:10 hr (dusk) and continued for approximately 15 min before the beetles commenced feeding. By comparison, no male beetles were observed flying or displaying sexual behavior around the control vials or the vials containing indole alone.

In 2001, similar results were recorded with large numbers of male beetles being caught in the traps baited with anisole (Figure 3). Male beetles were also caught in the traps baited with anisole and phenol. However, the numbers were smaller than those in the traps baited with anisole alone [$F(3,60) = 11.11$, $P < 0.001$]. This observation was interesting because it makes little sense for a female beetle to produce a compound that repels members of its own species. Explanations for this may include that the concentration of phenol emitted from the vials was excessive and, therefore, masked the attractiveness of the anisole. No beetles were caught in the traps baited with phenol alone.

The behavior of the male beetles in the presence of anisole was similar to that observed in males seeking a mate. The normal pattern is for males to emerge

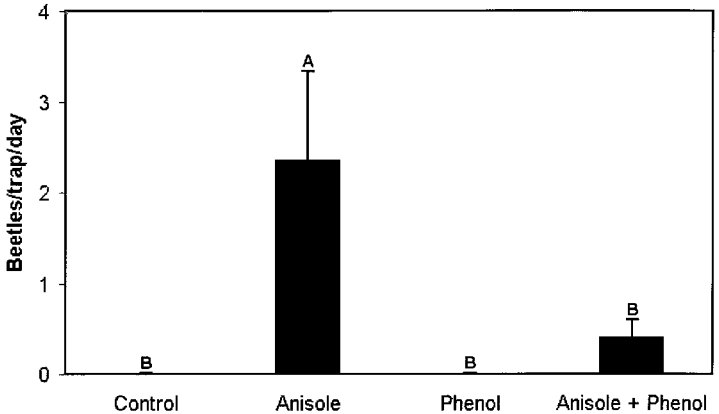


FIG. 3. Number of male *H. reynaudi* beetles attracted to vials containing anisole, phenol, and a mixture of anisole + phenol. No female beetles were caught. Bars marked with the same letter are not significantly different at $P < 0.05$ using least significant differences.

from the soil and fly around searching for calling females. If successful, copulation usually commences within five minutes of emergence from the soil. Males that are unable to find a mate continue to patrol in an attempt to find a calling female before settling to feed after approximately 15 min.

Although the number of females attracted to the candidate chemicals differed in 2000 [$F(3,36) = 3.55$, $P = 0.02$] (Figure 2), the number of females was low in all treatments. In 2001, no females were caught. Importantly, the behavior of female beetles was unchanged by the presence of the candidate compounds. In both the presence and absence of the candidate chemicals, female beetles emerged from the soil and commenced calling in an attempt to attract male beetles. Although mating usually commences within 5 min of emergence, females located close to the vials containing anisole were observed calling unsuccessfully for periods in excess of 10 min before ceasing, while males attempted to mate with either the vials containing anisole or each other less than 1 m away.

The field bioassay conducted at Mahbubnagar gave almost identical results to those obtained at ICRISAT. Large numbers of beetles (>200) were attracted to the trees baited with anisole, while low numbers of beetles were observed on the control and indole trees. Regular checks of the sex of the beetles flying around the trees baited with anisole showed that all of the beetles were male. Similarly, all of the beetles captured ($N = 47$) in the funnel trap baited with anisole were male.

Sex pheromones can be defined as compounds emitted by individuals of one sex that attract members of the opposite sex, resulting in the location of the emitter, and subsequently, mating (Baker, 1989; Tamaki, 1985). From our observations,

anisole can be regarded as a sex pheromone because it is released by females, and males show a clear sexual response to it. When vials containing anisole were placed on the trees, male beetles were observed orienting towards the vials after emerging from the soil. When they reached the vials, the beetles landed on or close to the vial and buzzed their wings vigorously before everting their genitalia and attempting to mate with either the vial or other male beetles in their vicinity.

Anisole has also been identified as the sex pheromone used by *H. consanguinea* (Leal et al., 1996), a species closely related to *H. reynaudi*, found in northern India. However, unlike *H. reynaudi*, where only male beetles were attracted to anisole, anisole was equally attractive to both male and female *H. consanguinea*, resulting in large aggregations of both male and female beetles. In northern India, this behavior is exploited for management of the pest, by spraying trees with insecticide prior to adult emergence (Yadava and Sharma, 1995), killing beetles of both sexes and reducing subsequent crop damage. More recently, this has been combined with the use of anisole to further concentrate the beetles and reduce the number of trees sprayed (Yadava, personal communication). On the basis of the data reported here, the prospects are limited for a similar approach with *H. reynaudi.*

From an evolutionary perspective, we believe that there are advantages for female beetles to release sex pheromones that attract members of the opposite sex only. Sex-specific pheromones may limit competition for males from other female beetles attracted by the semiochemical and, therefore, increase the probability of successful mating. This view is supported by Leal et al. (1996), who believe that, in the evolution of functional communication in scarab beetles, sex-specific signaling has evolved later than signals that attract beetles from both sexes. In this sense, *H. reynaudi* would be considered more advanced than *H. consanguinea* because the semiochemical used as a pheromone has developed a sex-specific function, attracting only male beetles.

The use of the same pheromone or complex of pheromones has previously been observed in closely related species from the subfamily Rutelinae. However, there are no similar reports in the melolonthines. Leal et al. (1994) observed that *Anomala albopilosa sakishimana* and *Anomala cuprea* shared the same pheromone. Similarly, Potter (1980) observed that *C. lurida* and *C. borealis* appeared to share the same pheromones. In both cases, reproductive isolation was maintained using either temporal or spatial means.

Although both *H. consanguinea* and *H. reynaudi* are endemic to India, there are no reports in the literature of them occurring sympatrically. As *H. reynaudi* is found in southern India and *H. consanguinea* is found in northern India, reproductive isolation is maintained through spatial separation, and as a result there is unlikely to be significant selective pressure for the pheromone system of either species to evolve further.

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IS DIMETHYLDECANAL A COMMON AGGREGATION PHEROMONE OF *Tribolium* FLOUR BEETLES?

LUDOVIC ARNAUD,[1,*] GEORGES LOGNAY,[2] MARJOLAINE VERSCHEURE,[2] LIONEL LEENAERS,[1] CHARLES GASPAR,[1] and ERIC HAUBRUGE[1]

[1]*Department of Pure and Applied Zoology*
[2]*Department of General and Organic Chemistry*
Gembloux Agricultural University
Passage des déportés 2, 5030 Gembloux, Belgium

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Abstract—Flour beetles are cosmopolitan and common pests in grain stores and flour mills. Their ability to exploit a wide variety of stored products has contributed to their status as major pests of stored food. Although it was previously reported that the same aggregation pheromone, 4,8-dimethyldecanal (DMD), is shared by three flour beetles species (*Tribolium castaneum*, *T. confusum*, and *T. freemani*), the volatiles released by the other *Tribolium* species associated with stored products have not yet been examined. In the present study, the volatiles produced by males and females of eight *Tribolium* species were examined by solid phase microextraction (SPME). SPME samples were analyzed by gas chromatography coupled to mass spectrometry (GC-MS). Experiments were conducted to identify volatiles emitted by the adults of different *Tribolium* species and to determine whether DMD is a common aggregation pheromone. We observed that DMD is not a common pheromone of the eight species tested, but is common to *T. castaneum*, *T. confusum*, *T. freemani*, and *T. madens*. Two other volatiles were detected, 1-pentadecene, which is shown here to be a common semiochemical of flour beetles, and 1,6-pentadecadiene, which was detected in five species (*T. audax*, *T. brevicornis*, *T. destructor*, *T. freemani*, and *T. madens*).

Key Words—Tenebrionidae, *Tribolium*, aggregation pheromone, epideictic pheromone, 4,8-dimethyldecanal, 1-pentadecene, SPME, GC-MS.

* To whom correspondence should be addressed. E-mail: arnaud.l@fsagx.ac.be

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INTRODUCTION

Worldwide losses of stored products due to postharvest insect attack are estimated to be 15% annually, and tremendous costs are involved in protecting commodities against insect infestations (Reichmuth et al., 1997). Tenebrionid flour beetles (*Tribolium* spp.) are common pests of numerous stored products. Their occurrence results in contamination and substantial economic damage due to loss of the product and a decrease in nutritional value (Sokoloff, 1974). Pesticides have been widely used to control pest infestations. However, many stored product pests are now resistant to many insecticides (Georghiou and Lagunes-Tejeda, 1991), and for some insecticides registrations have not been renewed due to safety hazards. Therefore, new methods of pest management are needed. The application of insect semiochemicals might represent a way to reduce the level of damages caused by stored product insects to acceptable levels.

Although many studies have examined chemical communication in flour beetles, only a few have identified the volatiles released by the adults and investigated their role as pheromones (Keville and Kannowski, 1975; Suzuki et al., 1975, 1984, 1987; Suzuki, 1980; Faustini and Burkholder, 1987). An aggregation pheromone, 4,8-dimethyldecanal (DMD), which is used in pheromone trapping systems (Hussain et al., 1994), has been identified in *T. castaneum* and *T. confusum* (Suzuki, 1980) and later also in *T. freemani* (Suzuki et al., 1987). Although other semiochemicals produced by flour beetles have been identified, currently only DMD is used to monitor flour beetles in stored product facilities.

The *Tribolium* genus can be subdivided in five species groups (Hinton, 1948; Sokoloff, 1974, Howard, 1987). Beetles of more than one species may occur in sympatry (Sinha and Watters, 1985). Interspecific attraction was observed between species belonging to the same or a different species group. However, cross-attraction was not reciprocal for all species tested (Suzuki et al., 1987, 1988a; Faustini et al., 1982). This phenomenon could lead to imperfect pre-reproductive isolation between some species. Moreover, within the *castaneum* species group, sterile hybrids are observed between *T. castaneum* and *T. freemani* (Nakakita et al., 1981). Hybrids are even observed between *T. audax* and *T. madens* (Sokoloff, 1972), two species belonging to distinct species groups (sensu Howard, 1987). Although *T. confusum* belongs to a different species group from *T. castaneum* and *T. freemani*, evidence indicates that *T. castaneum*, *T. confusum*, and *T. freemani* males produce the same aggregation pheromone (4,8-dimethyldecanal, DMD) (Suzuki, 1980; Suzuki et al., 1987). However, the response to the pheromone of the different species, and also of populations of the same species, depends upon the chirality of the molecules (Levinson and Mori, 1983; Suzuki and Mori, 1983; Barak and Burkholder, 1984/85; Boake and Wade, 1984; Suzuki et al., 1987). In addition, *Tribolium* species have other semiochemicals, such as quinones and cuticular hydrocarbons, in common (Lockey, 1978; Markarian et al., 1978; Wirtz

et al., 1978; Howard, 1987). Until now the role of these compounds and their potential utilization to monitor flour beetles has not been examined.

In the present study, we examined the production of volatile compounds by males and females of eight *Tribolium* species belonging to three species groups (sensu Howard, 1987): the *brevicornis* group: *T. audax* and *T. brevicornis*; the *castaneum* group: *T. castaneum*, *T. freemani*, and *T. madens*; and the *confusum* group: *T. anaphe*, *T. confusum*, and *T. destructor*. Our aims were to identify the volatile semiochemicals emitted by adults of the different species and to observe whether DMD was a common aggregation pheromone of flour beetles.

METHODS AND MATERIALS

Insects. Eight *Tribolium* species originating from different geographic areas were used (Table 1). Beetles were cultured in a dark incubator at $28 \pm 3^{\circ}$C and $65 \pm 5\%$ relative humidity with wheat flour and brewer's yeast (10/1, w/w) as rearing medium. Beetles were sexed as pupae by morphological characters (Ho, 1969) and maintained individually (to ensure their virginity) in small vials (5.5 cm^3) with 0.5 g of the medium. One-month-old adults were used in the experiments.

Volatile Collection: Sample Technique. To identify the volatiles emitted by virgin male and female *Tribolium*, we used a glass vial (10 cm^3) where one adult was placed with 0.2 g of wheat bran as feed. Vials were sealed with a rubber septum and kept in an incubator at $28 \pm 3^{\circ}$C and $65 \pm 5\%$ relative humidity. Volatile semiochemicals secreted by the adult were sampled after four days. For that purpose, glass vials were maintained at 35°C, and volatiles were sampled for 30 min by solid phase microextraction (SPME) with a polydimethylsiloxane (PDMS) fiber (100 μm, Supelco, Sigma-Aldrich, Belgium) previously conditionned for 1 hr at 250°C. Moreover, the SPME fiber was systematically reconditioned before each analysis. Since a preliminary set of 10 SPME samplings performed with males of *T. castaneum* revealed no qualitative differences, only three males and three

TABLE 1. *Tribolium* MCLAY (COLEOPTERA, TENEBRIONIDAE) SPECIES USED AND THEIR ORIGIN

Species	Common name	Origin, Year
T. anaphe (Hinton)	—	Nigeria, 1956
T. audax Halstead	American flour beetle	Canada, 1969
T. brevicornis (LeConte)	Giant flour beetle	USA, unknown
T. castaneum (Herbst)	Red flour beetle	USA, 1960
T. confusum DuVal	Confused flour beetle	Germany, 1967
T. destructor Uyttenboogaart	Dark flour beetle	Ethiopia, 1968
T. freemani (Hinton)	Kashmir flour beetle	Japan, 1980
T. madens (Charpentier)	Black flour beetle	Yugoslavia, 1959

females of each species were investigated. A vial with 0.2 g of wheat bran was used as control.

SPME and GC-MS: Analytical Method. GC-MS analyses were performed on a mass spectrometer Hewlett-Packard 5972 coupled to a Hewlett-Packard 5890 Series II gas chromatograph (splitless mode at 250°C) fitted with a Hewlett-Packard HP-5 MS column (30 m × 0.25 mm; 1 μm). The temperature program was from 40 to 200°C at 8°C/min and from 200 to 280°C at 20°C/min. Helium was used as carrier gas at 1.0 ml/min. Mass spectra were recorded in the EI mode at 70 eV (scanned mass range: 35–400 amu). The interface and the source were maintained at 280°C and 250°C, respectively. Identifications were performed by comparing the recorded mass spectra with those of the Wiley 275.L computed data base and by determination of the retention times and mass spectra interpretations of pure DMD (Biosystèmes, France) and 1-pentadecene (Sigma-Aldrich, Belgium). Quantification of DMD was carried out by integration of the peak areas by using an external standard method. For calibrations, solutions of known amounts of DMD in *n*-hexane were injected in analytical conditions identical to that of SPME analyses.

RESULTS

DMD is not a common semiochemical of the genus *Tribolium*; it is produced in *T. castaneum*, *T. confusum*, *T. freemani*, and *T. madens* (Table 2). Two other volatiles were detected, 1-pentadecene, which is shown here to be a common semiochemical of flour beetles, and 1,6-pentadecadiene, which was detected in five species (*T. audax*, *T. brevicornis*, *T. destructor*, *T. freemani*, and *T. madens*) (Table 3).

TABLE 2. PRODUCTION OF 4,8-DIMETHYLDECANAL (DMD) BY *Tribolium* MALES AND FEMALES[a]

Species	DMD (ng/beetle)	
	Male	Female
T. anaphe	nd[b]	nd
T. audax	nd	nd
T. brevicornis	nd	nd
T. castaneum	0.71 ± 0.12 ($N = 3$) a	nd
T. confusum	0.18 ± 0.01 ($N = 3$) b	nd
T. destructor	nd	nd
T. freemani	0.12 ± 0.02 ($N = 3$) b	nd
T. madens	0.22 ± 0.17 ($N = 3$) b	nd

[a] Three males and three females were sampled for each species. Volatiles were trapped with a SPME fiber during 30 min.
[b] nd = not detected. Values followed by the same letter are not significantly different (Tukey's test, $P > 0.05$).

TABLE 3. PRODUCTION OF 1-PENTADECENE AND 1,6-PENTADECADIENE BY *Tribolium* MALES AND FEMALES[a]

Species	1-Pentadecene			1,6-pentadecadiene	
	Male (N)	Female (N)	Estimated quantity (ng)	Male (N)	Female (N)
T. anaphe	2	3	0.73 ± 0.35	nd	nd
T. audax	3	2	0.46 ± 0.26	3	2
T. brevicornis	3	2	1.93 ± 0.28	3	2
T. castaneum	1	nd	0.21	nd	nd
T. confusum	1	1	0.49 ± 0.41	nd	nd
T. destructor	1	1	0.09 ± 0.05	nd	1
T. freemani	3	3	0.21 ± 0.07	1	3
T. madens	3	2	0.12 ± 0.01	3	2

[a] Three males and three females were sampled for each species. Volatiles were trapped with a SPME fiber during 30 min.
N = number of adults that releazed the compound, nd = not detected.

Typical chromatograms (total ion current) of the SPME sampling of the volatiles released by flour beetles are illustrated in Figure 1. The peaks at 16.15 min (retention time, t_R) and 18.70 min correspond to DMD and 1-pentadecene, respectively. Both the recorded mass spectra and retention times were identical to those of the authentic compounds. In addition, their fragmentation patterns were indistinguishable from those in the Wiley 275.L data base. Although the molecular ion (M^+ 184) of DMD has never been observed, a characteristic ion was obtained at m/z 140. It corresponds to a fragmentation resulting from the McLafferty rearrangement due to the β–cleavage of the aldehyde group.

An additional constituent (RRT = 1.016; 1-Pentadecene as reference) detected mainly in *T. brevicornis* and in lower proportions in *T. audax*, *T. destructor*, *T. freemani*, and *T. madens* showed a mass spectrum identical to that of *cis*-cyclododecene from the Wiley 275.L computed library. Nevertheless, the injection of a reference mixture of *cis*- and *trans*-cyclododecene (Fluka, Belgium) did not corroborate this interpretation. To establish the identity of the molecule, 250 flour beetles (*T. brevicornis*) were extracted with 50 ml peroxide-free diethyl-ether. After concentration to 500 μl under a gentle stream of nitrogen, the ethereal extract was submitted to GC-MS analysis. A well-resolved peak with both RRT (1.012) and fragmentation pattern similar to that observed during SPME analysis was observed. The molecular peak $M^+ = 208$ indicated a pentadecadiene isomer. The peak was attributed to 1,6-pentadecadiene (Suzuki et al., 1975; Wirtz et al., 1978; Howard, 1987). Although it was observed here in only five *Tribolium* species, it was previously observed as a common chemical of the same eight species examined here (Howard, 1987).

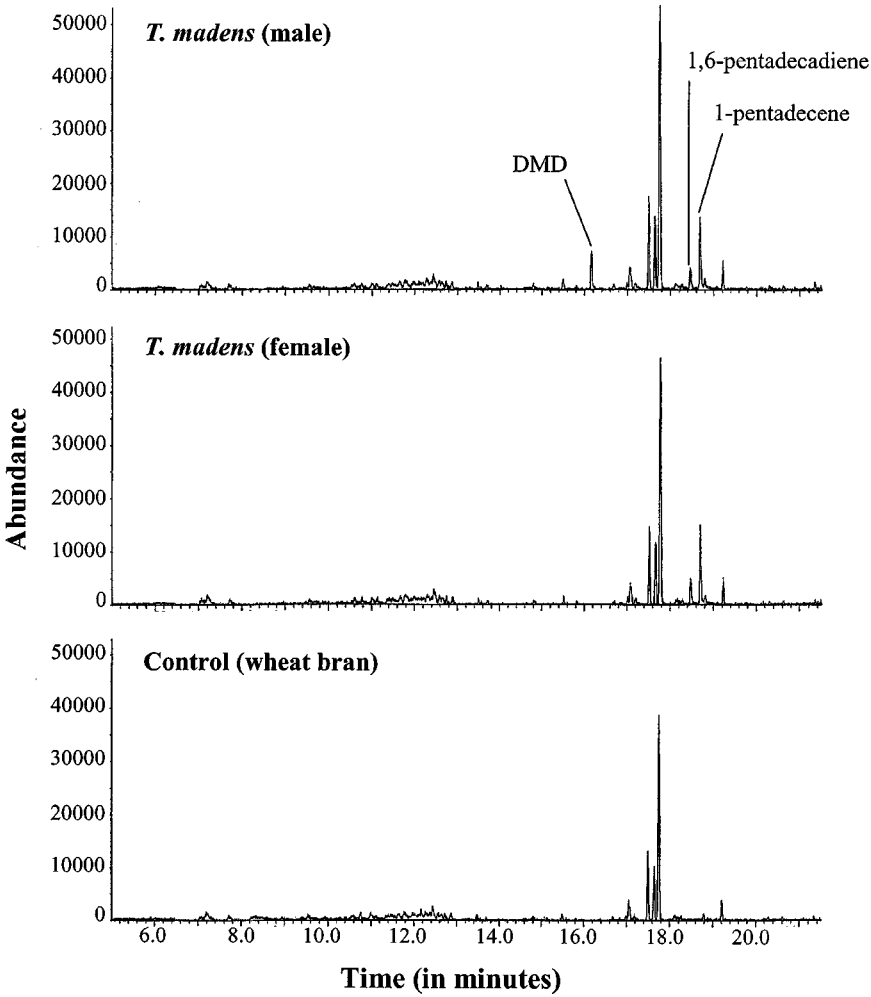


FIG. 1. Typical chromatogram (total ion current) of the PDMS SPME sampling of the volatiles released by male and female *Tribolium madens*. 4,8-Dimethyldecanal (t_R = 16.15 min), 1,6-pentadecadiene (t_R = 18.48 min), 1-pentadecene (t_R = 18.68 min). The other unidentified compounds are generated by the wheat bran.

The volatiles released by males and females of the eight species are shown in Tables 2 and 3. The values presented in the tables are an estimation of the quantities of the volatiles released by the adults by comparison of the area of the peak in insect volatiles to that of a known amount of DMD and of 1-pentadecene. Not all of the three semiochemicals were released by adults of each species. Furthermore, the species differed in the quantity of compounds they released.

DMD has been identified previously in *T. castaneum*, *T. confusum*, and *T. freemani* and is now reported for the first time in *T. madens* males. However, it was not secreted by female beetles. One-way ANOVA showed that males of the three species differ in the quantity of DMD secreted ($F_{3,8} = 6.80$, $P = 0.014$). However, there were no significant differences among *T. confusum*, *T. freemani*, and *T. madens* (Tukey's test, $P > 0.05$).

We observed that 1-pentadecene was released from all species (Table 3). However, it appeared that this compound was not secreted by all individuals of a species and that the production was highly variable among individuals. The 1,6-pentadecadiene ($t_R = 18.48$ min) was detected in adults of *T. audax*, *T brevicornis*, *T. destructor*, *T. freemani*, and *T. madens*, but was not found systematically in each replicate of each species.

DISCUSSION

Since the identification of the same aggregation pheromone for three *Tribolium* species (Suzuki, 1980; Suzuki et al., 1987), DMD has been considered a common volatile in flour beetles. A synthetic pheromone mostly composed of the (4*R*,8*R*) isomer was, thus, commonly used to trap the confused and the red flour beetles in stored product commodities. Indeed, we observed that DMD is not a common volatile of *Tribolium* flour beetles. It was produced only by males of four of the eight species tested.

Hussain (1993) observed that, in *T. castaneum*, DMD was released also by young females (<2 days old) and by larvae and pupae. We used 1-month-old insects in our experiments. This could explain the absence of DMD production by females. The quantities of DMD detected with our sampling method are much smaller than those reported by Hussain (1993) and Bloch Qazi et al. (1998). Working with a Super-Q column, these authors had observed that, on average, a single *T. castaneum* male produces 633 ng/24 hr and 163 ng/48 hr, respectively. In high-density conditions (2000 males in a 500-ml Erlenmeyer flask for 40 days), Suzuki and Sugawara (1979) observed that about 3.17 ng of DMD was secreted by one adult per day. Hussain (1993) observed that DMD production decreases while population density increases.

Ethyl- and methyl-benzoquinones are secreted by adult flour beetles (Alexander and Barton, 1943; Markarian et al., 1978; Howard and Mueller, 1987; Hussain, 1993; Pappas and Wardrop, 1996). Quinones were trapped with a Super-Q column connected to an aeration chamber containing one adult red flour beetle by Hussain (1993). We did not trap any quinones in our experiments. Quinones are released by adult flour beetles in stressful (Tschinkel, 1975) or overcrowded conditions (Faustini and Burkholder, 1987). Either the PDMS fiber (apolar phase) was not suitable to trap quinones (polar molecules), or our sampling conditions were not stressful to the insects. In order to differentiate between these hypotheses,

we used a Carboxen-PDMS SPME fiber, which is suitable for polar compounds. Moreover, vials containing one *T. castaneum* male (unstressful conditions) or 25 unsexed *T. castaneum* adults (stressful conditions) were sampled. Sampling was conducted as described above. By using the PDMS fiber, DMD was detected in both kind of samples, but quinones were not. Ethyl- and methyl-benzoquinones were easily detected with the Carboxen-PDMS fiber in the stressful conditions (25 adults), but no detectable amounts of quinones were observed in the unstressful conditions (1 male). DMD was not detected with that fiber. Both hypotheses can, thus, explain our results: PDMS fiber is not suitable to detect quinones, and stressful conditions are necessary to bring about the release of quinoid compounds. An interesting observation is that, contrary to Hussain (1993), DMD was detected even in stressful conditions.

The unsaturated hydrocarbon 1-pentadecene has been observed in studies examining cuticular lipids and defensive secretions of flour beetles (Keville and Kannowski, 1975; Tschinkel, 1975; Suzuki et al., 1975, 1988b; Lockey, 1978; Markarian et al., 1978; Wirtz et al., 1978; Hebanowska et al., 1990). In *T. confusum*, 1-pentadecene was believed to bring the sexes together and induce copulation because, although it was produced by males and females, only males appeared to be affected (Keville and Kannowski, 1975). However, it was first hypothesized that 1-pentadecene was used to facilitate the absorption of quinones by *Tribolium* enemies (Endt and Wheeler, 1971). Suzuki et al. (1975) observed that 1-pentadecene was repellent to *T. castaneum* and *T. confusum* adults at very low concentrations (0.1–10 μg/21-mm disk). Considering the amount of 1-pentadecene used by Keville and Kannowski (1975) (2–2.5 mg/6 mm disk) and the quantity of 1-pentadecene secreted by adults of the confused flour beetle (3 μg/beetle/3 hr) (Endt and Wheeler, 1971), the repellent hypothesis seems to be more likely. We, thus, hypothesized that 1-pentadecene may function as an epideictic (spacing) pheromone to flour beetles and a defensive secretion against *Tribolium* enemies. However, its exact function is not yet established. In this study, we observed that 1-pentadecene was produced by males and females of all species tested, except *T. castaneum* males. However, this volatile has been previously observed in the male red flour beetle (Howard, 1987; Hussain, 1993). Therefore, 1-pentadecene could be considered a common volatile of *Tribolium* flour beetles.

Our findings increase knowledge on chemical communication in *Tribolium* flour beetles and have implications in integrated pest management of these stored-products insects. Hitherto, DMD has been considered a common aggregation pheromone of flour beetles and is used in pheromone traps (Barak and Burkholder, 1984/85; Hussain et al., 1994). However, in flour beetles, the efficiency of the pheromone trap depends on the isomeric composition of the synthetic compound, on the target species (Levinson and Mori, 1983; Suzuki and Mori, 1983; Barak and Burkholder, 1984/85; Suzuki et al., 1984, 1987), and even on the population of the species (Boake and Wade, 1984). Although it was not produced by every adult, we observed that 1-pentadecene was a common volatile to flour

beetles. Therefore, it could be used as an alternative semiochemical to DMD, which is restricted to some species. Behavioral experiments are still needed to determine its precise role in *Tribolium* chemical communication and to determine its potential use in monitoring and/or controlling flour beetles in stored products. If its repellent role is confirmed, 1-pentadecene could instead be used as a protectant of stored products against *Tribolium* flour beetles.

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FEMALE SEX PHEROMONE POLYMORPHISM IN ADZUKI BEAN BORER, *Ostrinia scapulalis*, IS SIMILAR TO THAT IN EUROPEAN CORN BORER, *O. nubilalis*

YONGPING HUANG,[1,2] TAKUMA TAKANASHI,[2] SUGIHIKO HOSHIZAKI,[2] SADAHIRO TATSUKI,[2] and YUKIO ISHIKAWA[2,*]

[1]*Laboratory of Applied Entomology*
Graduate School of Agricultural and Life Sciences
The University of Tokyo
Tokyo 113-8657, Japan

[2]*Present address: Shanghai Institute of Plant Physiology & Ecology*
Academia Sinica, 300 Fenglin Road
Shanghai 200032, China

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Abstract—Individual analysis of the female sex pheromone of the adzuki bean borer, *Ostrinia scapulalis*, has shown that the sex pheromone of this species comprised (*E*)-11-tetradecenyl acetate (E11–14:OAc) and (*Z*)-11-tetradecenyl acetate (Z11–14:OAc) at variable blend ratios. The pheromone blend could be tentatively categorized into three types with respect to the proportion of *E*11–14:OAc: E type (94–100%, median 99.2%), Z type (0–16%, median 3.0%), and intermediate type (I type, 48–85%, median 63.7%). In addition to the identity of components, the blend ratios in the three types were similar to those of the E strain, Z strain, and hybrid of the European corn borer, *O. nubilalis*, respectively. This finding suggests that two closely related but morphologically distinct species, *O. scapulalis* and *O. nubilalis*, share almost the same sex pheromone communication systems. The significance of this similarity in the two sibling species is discussed.

Key Words—*Ostrinia scapulalis*, sex pheromone, (*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, *Ostrinia nubilalis*, polymorphism.

* To whom correspondence should be addressed. E-mail: ayucky@mail.ecc.u-tokyo.ac.jp

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INTRODUCTION

The adzuki bean borer *Ostrinia scapulalis* (Lepidoptera: Crambidae) is one of the seven *Ostrinia* species currently found in Japan and is an important pest of the adzuki bean plant *Vigna angularis*, cowpea *V. sinensis*, and hop *Humulus lupulus*. *Ostrinia scapulalis* is morphologically similar to the European corn borer *O. nubilalis* but is distinct in the following aspects: (1) The middle tibia of *O. scapulalis* males are large, while those of *O. nubilalis* males are small (Mutuura and Munroe, 1970). (2) *O. nubilalis* is found mainly in Europe and North America but not in Japan, while *O. scapulalis* is found mainly in Asia including Japan and Eastern Europe (Frolov, 1998; Ishikawa et al., 1999). (3) Although both *O. nubilalis* and *O. scapulalis* are polyphagous, the host range of *O. scapulalis* is much narrower. In particular, *O. scapulalis* does not feed on maize and other Gramineae plants (Hattori and Mutuura, 1987), while *O. nubilalis* is an important pest of maize. In Asia, another congener, *O. furnacalis* (Asian corn borer), occupies the niche of maize pest.

Huang et al. (1997) identified the female sex pheromone of *O. scapulalis* from a population collected at Matsudo, located in the central part of Japan. The pheromone was found to be a 3:97 blend of (*E*)- and (*Z*)-11-tetradecenyl acetate, the same blend as that of the Z-strain European corn borer *O. nubilalis* (Klun et al., 1973, 1975). During the course of a continuing study, however, we found a case where progenies of a female *O. scapulalis* collected at the same location produced a pheromone of opposite blend ($E:Z = 99:1$), similar to that of the E-strain *O. nubilalis* (Kochansky et al., 1975; Klun et al., 1975). Furthermore, we found a case where a single mother of *O. scapulalis* collected at Matsudo produced female offspring of variable pheromone blends. We report here these findings on the variation of the sex pheromone in *O. scapulalis*, in comparison with that of the European corn borer *O. nubilalis*.

METHODS AND MATERIALS

Insects. Since morphological identification of *Ostrinia* female moths to species level is impractical, the following procedure was undertaken to prepare *O. scapulalis* families: Wild female moths of *Ostrinia* spp. were captured with an insect net at Matsudo (35.8°N, 139.9°E), Japan in September 1995. They were placed individually in plastic cups (9 cm diam. × 4.5 cm) with a cotton pad soaked with 5% sucrose solution, brought to the laboratory, and kept in an environmental chamber maintained at 25°C with a 15L:9D regime. Most of the females produced fertile eggs on the surface of the cups within a few days. Eggs produced by each female were treated separately as one family, with the mother's identity noted. Larvae were reared on a general diet for insects (Insecta LF, Nihon Nosan Co., Yokohama). The pupae obtained were sexed based on the morphology of terminal abdominal segments and maintained separately. Identification of the offspring

to species was based on the morphology of male moths (Mutuura and Munroe, 1970; Y. Yoshiyasu, unpublished). More than half the families were found to be *O. scapulalis*, the remainder being *O. furnacalis* and *O. orientalis*.

Sex Pheromone Analyses. The abdominal tip, which includes a sex pheromone gland, of a 3-day-old female moth was cut during the calling period in the late scotophase and immersed for 30 min in 5 μl redistilled hexane. The extract was concentrated to less than 2 μl under a dry nitrogen stream and analyzed with a gas chromatograph (Shimadzu GC 14-B) fitted with a split/splitless injector, a flame ionization detector, and a fused silica capillary column (DB-Wax, 30 m $\times$ 0.25 mm ID, J&W Scientific). The oven temperature was maintained at 100°C for 2 min, and programmed at 5°C/min to 240°C. The carrier gas was nitrogen, and the splitless injection time was 2 min. The ratio of the two pheromone components was calculated based on the peak areas.

RESULTS

Pheromone gland extracts from individual *O. scapulalis* females comprised E11–14:OAc and Z11–14:OAc at various blend ratios. The patterns of variation in pheromone blend differed from family to family among the eight examined (Figure 1). All females in two families (MD5202 and MD5208) produced a Z11–14:OAc-dominant pheromone (hereafter referred to as Z type). In contrast, almost all females in MD5304 produced an E11–14:OAc-dominant pheromone (E type). Moreover, analysis of females in families MD5211, MD5301, and MD5305 indicated females that produce pheromones with blends intermediate to the Z and E types (I type). Within the families analyzed, MD5300, MD5301, and MD5305 comprised the three pheromone types, MD5205 and MD5211 comprised Z and I types, and MD5304 comprised E and I types (Figure 1).

By comparisons of the pheromone blend distribution patterns in all families, the proportion of E11–14:OAc in Z type was estimated to range from 0 to 16% (median 3.0%, but see below). Similarly, the percentage range of E11–14:OAc in E type was estimated to be very narrow: 94–100% (median 99.2%). The proportion of E11–14:OAc in the I type appeared to extend from 48 to 85%, with a median at 63.7%. Although a female in MD5300 produced a pheromone blend near 35% E11–14:OAc, we cannot assign this blend either as Z type or I type from the available information.

DISCUSSION

The sex pheromone polymorphism in *O. scapulalis* appears to be similar to that in a sibling species, the European corn borer *O. nubilalis*. Since the discovery of extreme sex pheromone polymorphism in *O. nubilalis* in 1970s, scientists from various research fields have utilized this insect as a model for their studies. Thus,

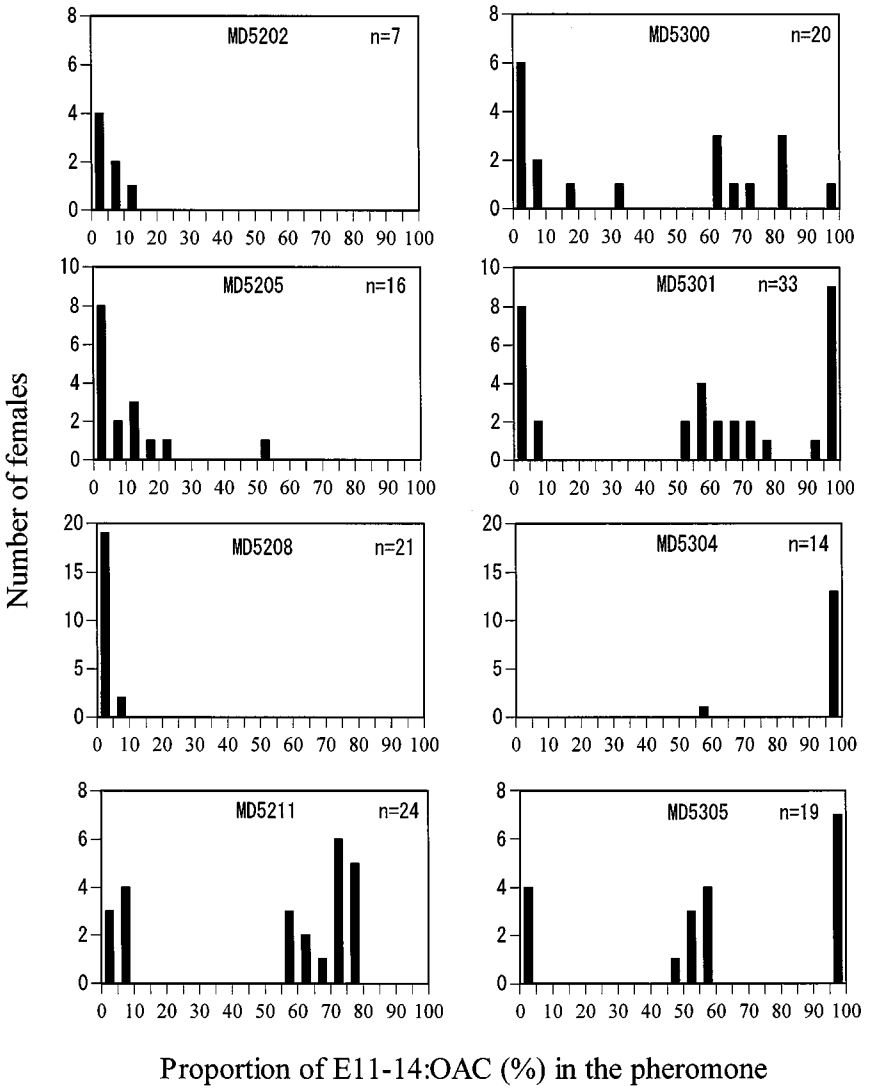


FIG. 1. Female sex pheromone blend of daughters from eight families of the adzuki bean borer, *Ostrinia scapulalis*. The female sex pheromone of *O. scapulalis* comprised (*E*)- and (*Z*)-11-tetradecenyl acetates (E11– and Z11–14:OAc), and the blend ratio is expressed by the proportion of E11–14:OAc.

understanding of various features of this phenomenon, e.g., genetic control of the sex pheromone blend in females and that of behavioral responses in males (Klun and Maini, 1979; Roelofs et al., 1987; Linn et al., 1997), have increased greatly.

The patterns of variation in the pheromone blends in *O. scapulalis* families strongly suggest that the variation has a genetic basis. The blend of pheromones in *O. nubilalis* is controlled by two codominant alleles ***E*** and ***Z*** at a single autosomal locus (Klun and Maini, 1979; Roelofs et al., 1987). The percentage ranges of E11–14:OAc in the E-type and Z-type *O. scapulalis* are similar to those in the corresponding two types in *O. nubilalis* (genotypes are ***EE*** and ***ZZ***, respectively). Moreover, the percentage range of E11–14:OAc in the I-type *O. scapulalis* (48–85%) is also similar to that of the hybrid *O. nubilalis* (***ZE***). It is, therefore, likely that the pheromone blend in *O. scapulalis* is under the same genetic control as that in *O. nubilalis*. This has to be confirmed through the establishment of pure *O. scapulalis* cultures of the Z and E types and crossing experiments that use these cultures.

The geographic variation in frequency of the pheromone types in natural *O. scapulalis* populations is of interest. The *Ostrinia nubilalis* Z strain predominates throughout Europe and North America, while the E strain is observed in southern Switzerland and northern Italy in Europe, and in northeastern North America (Klun et al., 1975; Anglade et al., 1984; Peña et al., 1988). Hybrid *O. nubilalis* are found only in limited regions where the two strains occur sympatrically (Roelofs et al., 1985; Peña et al., 1988; Klun and Huettel, 1988; Glover et al., 1991). In contrast, three pheromone types of *O. scapulalis* are found sympatrically in most regions of Japan (Takanashi, unpublished data), and in our main research area at Matsudo, the I type appears to be dominant.

Since *O. scapulalis* and *O. nubilalis* share practically identical sex pheromone communication systems, hybridization between the species would be expected if they occur sympatrically. Available distribution information on *O. scapulalis* and *O. nubilalis* suggests that although the two species occur largely allopatrically, cooccurrence is observed in some regions, mainly in Eastern Europe (Frolov, 1998; Ishikawa et al., 1999). To make the situation more complex, it has been suggested that populations of *O. nubilalis*, and probably those of *O. scapulalis*, are substantially differentiated geographically and in their use of host plants (Frolov, 1995, 1998). In laboratory experiments, hybridization between *O. nubilalis* and *O. scapulalis* occurred only in certain combinations of the parental populations (Frolov, 1994). Therefore, it does not seem relevant to discuss reproductive isolation between the two species as a whole; rather, careful investigations of geographic and host-associated differentiation, as well as sex pheromone types, in the populations are required.

It would be of interest to know the origin of similarity in the female sex pheromones of *O. scapulalis* and *O. nubilalis*: whether the pheromone polymorphisms in the two species were acquired independently or not. To answer this

question, we must clarify the sex pheromones and phylogenetic relationships among all extant *Ostrinia* species worldwide. Analyses of sex pheromones and phylogeny of all *Ostrinia* species in Japan, is currently in progress in our laboratory (Ishikawa et al., 1999; Kim et al., 1999), and this should contribute to the understanding of this interesting phenomenon.

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EXPERIMENTAL EVIDENCE OF AN AGE-SPECIFIC SHIFT IN CHEMICAL DETECTION OF PREDATORS IN A LIZARD

MEGAN L. HEAD, J. SCOTT KEOGH,* and PAUL DOUGHTY

School of Botany and Zoology
Australian National University
Canberra, ACT 0200, Australia

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Abstract—The risk posed by predation is one of the most fundamental aspects of an animal's environment. Avoidance of predators implies an ability to obtain reliable information about the risk of predation, and for many species, chemosensory cues are likely to be an important source of such information. Chemosensory cues reliably reveal the presence of predators or their presence in the recent past. We used retreat site selection experiments to test whether the Australian scincid lizard *Eulamprus heatwolei* uses chemical cues for predator detection and avoidance. Both adult and juvenile lizards were given the choice of retreat sites treated with scents from invertebrate predators, as well as sympatric and allopatric snake predators. Some of the snake predators were known to eat *E. heatwolei*, while others did not pose a predation threat. All invertebrate predators posed a risk to juveniles, but not adults because of their size. We found that juvenile *E. heatwolei* avoided predator odors more strongly than adults. Juveniles avoided both invertebrate predators and snakes, and the strongest response was toward the funnelweb spider, the only ambush predator used in this experiment. This result may demonstrate the importance of predator ecology in the evolution of predator detection mechanisms, with chemical cues being more useful in detecting sedentary predators than active predators. Adult lizards showed no avoidance behavior toward predator odors. This result suggests an age specific shift in predator avoidance behavior as lizards get older and become too large for many predators. However, adults showed no response to the odor from the red-bellied black snake, a known predator of adult *E. heatwolei*. This finding further demonstrates the importance of predator ecology when examining communication between predators and prey. Chemical cues, which are persistent long after predators have vacated the area, may not be useful in detecting the red-bellied black snake, a wide-ranging active forager.

* To whom correspondence should be addressed. E-mail: scott.keogh@anu.edu.au

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Key Words—Behavioral ecology, chemical ecology, predator avoidance, ontogenetic shift, reptile, lizard.

INTRODUCTION

Recognition of and response to predator odors is common and widespread throughout the animal kingdom. Avoidance of predator chemical cues has been demonstrated in mollusks, arthropods, fish, amphibians, mammals, and to a much lesser extent in echinoderms, reptiles, and birds (Kats and Dill, 1998). While most prey are capable of using multiple sensory mechanisms for predator detection, there appear to be important advantages to using chemical cues (Kats and Dill, 1998). Chemical cues reliably reveal the presence of predators (or their presence in the immediate past) without the need to actually confront the predator (Van Damme and Castilla, 1996). The ability to detect predators chemically is particularly advantageous for organisms whose activity patterns or environment preclude the effective use of other sensory systems (Petranka et al., 1987).

Chemical identification of predators by lizards has received little attention. Given their well-developed chemosensory systems, the potential for chemosensory-based predator avoidance behavior appears to be considerable (Dial, 1990), although Simon et al. (1981) found no evidence for chemical detection of snake predators by the phrynosomatid *Sceloporus jarrovi.* Detection of predators via chemical cues has been reported in a number of other lizard species. These studies have shown that these lizards can detect snake predator odors, as well as distinguish between the odors of predatory snakes and nonpredatory snakes (e.g., Thoen et al., 1986; Dial et al., 1989; Cooper, 1990; Dial, 1990; Dial and Schwenk, 1996; Van Damme and Quick, 2001). Differences among populations in response to snake predator odors have been demonstrated for a number of lizards. In some species, populations living sympatrically with predators respond more vigorously to predator scent than do populations living allopatrically with the same predator (e.g., Thoen et al., 1986; Van Damme and Castilla, 1996; Downes and Shine, 1998; Downes and Adams, 2001). Moreover, age related differences in thermoregulatory behavior in response to snake predator odors have been reported in *Lacerta vivipara*, with juveniles basking less than adults when exposed to the scent of a predator (Van Damme et al., 1995). Other studies have examined lizard response to predators at different ages (e.g., Phillips and Alberts, 1992). These studies, however, concentrated on whether chemical detection of predators is innate or learned, and did not focus on age specific shifts in predator avoidance behavior.

The southern water skink, *Eulamprus heatwolei*, is a medium-size (<110 mm snout-vent length, SVL) diurnal scincid lizard that occurs in southeast Australia. They are insectivorous and can often be found basking on logs on the forest floor of sclerophyll riparian zones. To examine differences in the responses of adult and juvenile *Eulamprus heatwolei* to predator odors, we conducted retreat site

selection experiments involving a range of chemical stimuli from potential snake and invertebrate predators. Snake predator odors were obtained from snake species that are known to eat lizards and are sympatric with our population of *E. heatwolei*. To test whether lizards respond to snake odor in general or only those that naturally pose a predation threat, we also included two snake species that are allopatric with our lizard population and are not known to eat *E. heatwolei*.

In all previous studies on chemosensory based antipredator behavior, the chemical stimuli have been derived from snakes alone. No studies on lizards have used chemical stimuli from invertebrate predators, even though many arthropods are predators of small lizards, and several arthropods and lizards may be involved in cross predation (where vertebrates are sequentially the prey and later the predator of the same species of invertebrate) (McCormick and Polis, 1982). By using invertebrate predators, we have the opportunity to examine age-specific shifts in antipredator behavior as well as to examine the difference in lizard responses to vertebrate and invertebrate predator odors by both adults and juveniles. We predicted that adult lizards would not avoid invertebrate odors and may even be attracted to them, whereas juveniles should avoid these odors, as they pose a predation threat. All lizards were expected to avoid odors from sympatric, but not allopatric snakes.

METHODS AND MATERIALS

Lizards were collected in late September and early October 2000 from the Tidbinbilla Nature Reserve (Canberra, ACT, Australia, 800 m elevation). This capture period occurred immediately after spring emergence before mating began. Juveniles were caught from the field at the same time and from the same locations as adults. Based on their size, juveniles were most likely born the previous season, making them approximately 1 year old. The lizards were brought back to a laboratory at the Australian National University where they were measured, weighed, and their sex determined via hemipene eversion.

Adult lizards were housed individually in plastic boxes (43L $\times$ 32W $\times$ 23H cm) and juveniles four or five to a box. All boxes contained a bark substrate, water bowl, and a ceramic tile for shelter. A 40-W light bulb was placed at one end of the box to allow lizards to thermoregulate naturally. The light bulb was turned off at night so they experienced a natural photoperiod. During the day, when the light bulb was on, temperatures ranged from 33°C under the light bulb to 25°C at the opposite end of the cage. All lizards were provided with food (*Tenebrio* larvae and dog food) and water ad libitum.

Lizards were tested with seven potential predator species, including four snakes and three invertebrates, as well as an odorless control. Species were chosen in order to test lizard response against a range of predators that presented different threats (Table 1). Three species of elapid snakes were used. *Pseudechis*

TABLE 1. POTENTIAL PREDATORS USED IN RETREAT SITE CHOICE EXPERIMENTS AND THREAT THEY POSE TO ADULT AND JUVENILE *Eulamprus heatwolei*

Predator	Sympatric with *E. heatwolei*	Main diet	Threat		References
			Juveniles	Adults	
Red-bellied black snake (*Pseudechis porphyriacus*)	Y	Frogs, small lizards,[a] small mammals	Major	Major	Shine, 1987; Swan, 1990
White-lipped snake (*Drysdalia coronoides*)	Y	Small lizards,[a] frogs	Major	None	Shine, 1981; Swan, 1990
Small-eyed snake (*Rhinocephalus nigrescens*)	N	Small lizards,[a] frogs, snakes	None	None	Shine, 1984; Swan, 1990
Childrens' python (*Liasis maculosus*)	N	Small mammals, small lizards	None	None	Shine and Slip, 1990; Swan, 1990
Centipede (Scolopendromorpha)	Y	Invertebrates, small vertebrates	Major	None	Butler, 1970; personal observation
Funnelweb spider (*Hadronyche* sp.)	Y	Invertebrates, small vertebrates	Major	None	Mascord, 1980; personal observation
Huntsman spider (Sparrasidae)	Y	Invertebrates, small vertebrates	Minor	None	Daniels and Heatwole, 1984; personal observation

[a] The predator has been recorded to eat *Eulamprus* species.

porphyriacus (red-bellied black snake) is a major threat to both adults and juveniles (Shine, 1987). *Drysdalia coronoides* (white-lipped snake) is a major threat to juveniles, but not adults because adults are too large for them to eat (Shine, 1981). *Rhinocephalus nigrescens* (small-eyed snake) does not occur at our study site, but is known to eat *Eulamprus* spp. elsewhere (Shine, 1984; Swan, 1990). It can be regarded as an elapid control to test whether *E. heatwolei* are responding to cues of actual predators and not just a general elapid odor. *Liasis maculosus* (children's python) preys mostly on mammals and sometimes on lizards. It does not overlap *E. heatwolei* in distribution and should not pose a threat (Shine and Slip, 1990; Swan, 1990). Thus, this snake can be used as a snake control to test whether *E. heatwolei* are responding to actual predator odors or a general snake odor.

A number of invertebrates have been recorded to prey on skinks (McCormick and Polis, 1982; Daniels and Heatwole, 1984). However, there are no reports on whether lizards are able to detect invertebrate predators via chemical cues. We tested adult and juvenile *E. heatwolei* with the odors of three sympatric invertebrates that are common at our study site and have been reported to eat small skinks (Daniels and Heatwole, 1984). They all pose a threat to juveniles, but not adults. Scolopendromorpha (centipedes) are active foragers that live under moist logs. *Hadronyche* sp. (funnelweb spiders) are ambush foragers that build their funnels only in moist rotting logs. Sparassidae (hunstman spiders) are generally arboreal, but sometimes forage on the forest floor.

This experiment was designed to test whether adult and juvenile *E. heatwolei* could detect predators via chemical cues and whether there was any difference in the way the two size (and age) classes of lizards responded to predator odors. A total of 14 adult (87–96 mm SVL) and 14 juvenile (43–61 mm SVL) lizards were tested for 12 nights with various combinations of predator odors. The experimental design consisted of a linked paired-comparison design (David, 1988, p. 100). This design allowed us to balance the experiment by judges as well as by comparisons. The experiment was repeated twice for adults and juveniles, for a total of 24 nights. Each pair of odors was tested six times, and for any two lizards, there are four pairs of odors that are compared by both lizards. Each night, lizards were removed from their home boxes and placed in plastic test boxes at 17:00 hr. Four hours later, the tile under which a lizard was sleeping was recorded. Trials were run in two 12-day blocks, the first from January 25 to February 5 and the second from March 1 to 12.

Test boxes ($30 \times 15 \times 7$ cm) contained a bark substrate and two retreat sites. Retreat sites consisted of two ceramic tiles (11×11 cm) placed at either end of a test cage. Tiles were raised 1 cm off the bottom of the cage with sticks to allow lizards to crawl underneath them. A paper towel treated with the odor of one of the seven predator treatments or the control was placed under each tile. Paper towels were placed on the floor of the predators' cage for three days to obtain their odors. Paper towel used for the odorless control was handled in the same way as

for predators except that it was not placed with a predator prior to being used. Cages and tiles were washed with detergent (Morning Fresh) and dried between all trials.

To test whether preferences were the same for both size classes, we compared the fit of a model allowing different preferences for both size classes and a model with the same preferences for both size classes. If the model allowing different preferences fit the data better than the alternative model, then the size classes were analyzed separately by using the Bradley-Terry model. The Bradley-Terry model is a parametric test that allows treatments to be ordered according to preference and is specifically designed for experiments consisting of paired comparisons (David, 1988). Likelihood ratio tests were used to determine whether the data conformed to the Bradley-Terry model and whether the Bradley-Terry model fit better than a no-preference model. If the Bradley-Terry model fits the data better than the no preference model, then it shows that there were strong preferences between the treatments (De'ath and Moran, 1998). A model of no preference also was tested against the data. If both models fit the data, then the model of no preference was retained.

Our analysis represents a novel application of the Bradley-Terry model. Additionally, this model is more appropriate for our analysis than statistics such as chi-square goodness of fit tests. This model uses information from all comparisons within an experiment, simultaneously, rather than information from each of the comparisons separately (Cowling, Head, and Stapley, personal observation).

RESULTS

A significant difference existed between adult and juvenile avoidance of predators ($\chi^2 = 23.469$, $df = 7$, $P \leq 0.005$). Juveniles avoided most of the treatments, while adults showed no preference among treatments (Figure 1). For the adults, the no-preference model fit the data just as well as the Bradley-Terry model (no preference: $\chi^2 = 8.857$, $df = 7$, $P = 0.263$; Bradley-Terry: $\chi^2 = 23.919$, $df = 21$, $P = 0.296$), therefore, the no preference model was retained. This result indicates that either adults could not detect the odors of potential predators, or if they could, they did not respond (Figure 1a). For juveniles, the no preference model did not fit the data as well as the Bradley-Terry model ($\chi^2 = 29.059$, $df = 7$, $P < 0.001$), whereas the Bradley-Terry model fit the data well ($\chi^2 = 14.557$, $df = 21$, $P = 0.845$), indicating a well-ordered hierarchy of juvenile preferences. Juveniles preferred tiles treated with the odor of the small-eyed snake over the odorless control, while all other vertebrate and invertebrate predator odors were avoided in comparison to the control (Figure 1b). The odor avoided most was that of the funnelweb spider. The probability of juveniles choosing the control over the funnelweb spider was 0.835 (Table 2).

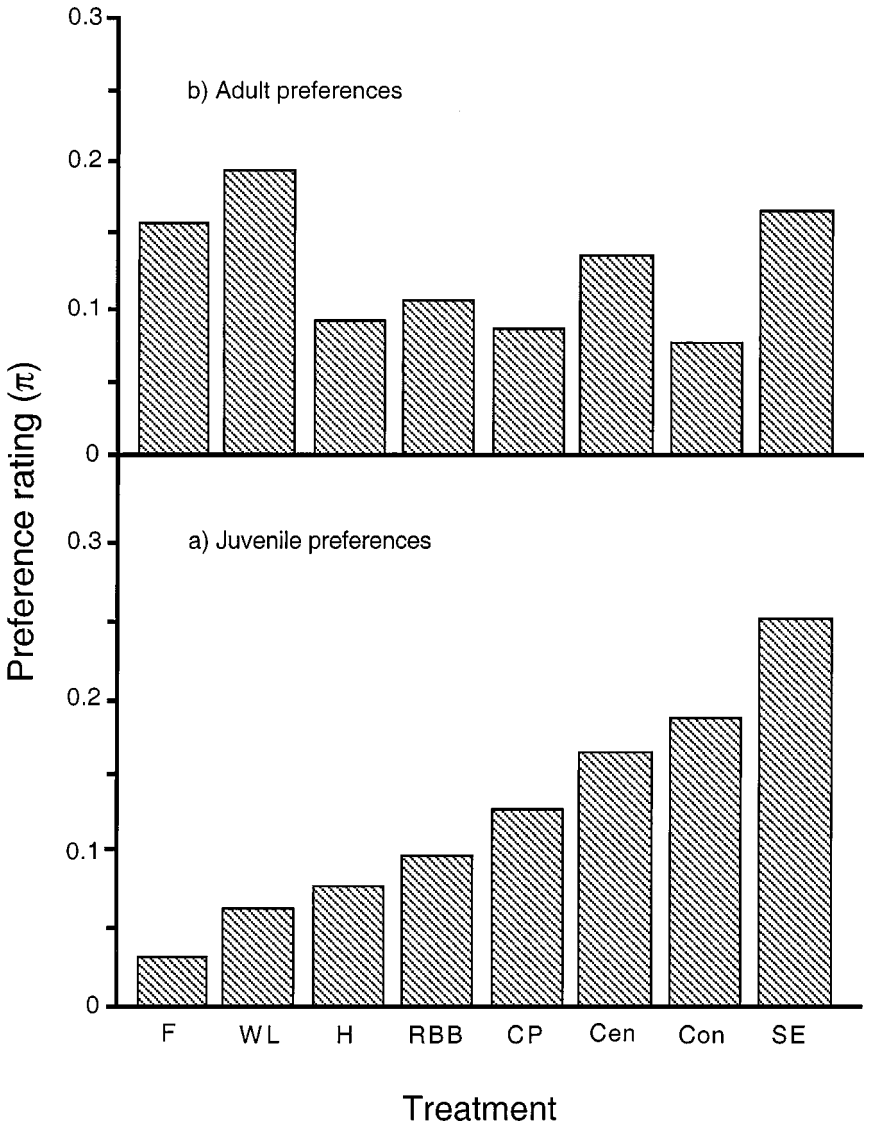


FIG. 1. Juvenile (a) and adult (b) preference ratings (π) obtained from the Bradley-Terry model. F = funnelweb spider, WL = white-lipped snake, H = huntsman spider, RBB = red-bellied black snake, CP = childrens' python, Cen = centipede, Con = odorless control, SE = small-eyed snake.

TABLE 2. PROBABILITY OF JUVENILES CHOOSING TREATMENT 1 OVER TREATMENT 2 AS ESTIMATED FROM BRADLEY-TERRY PREFERENCES

	Treatment 1							
Treatment 2	Red-bellied black snake	Small-eyed snake	White-lipped snake	Childrens' python	Huntsman spider	Funnelweb spider	Centipede	Odorless control
Red-bellied black snake	—	0.720	0.405	0.569	0.446	0.248	0.628	0.656
Small-eyed snake	0.280	—	0.209	0.339	0.238	0.113	0.397	0.425
White-lipped snake	0.595	0.791	—	0.557	0.542	0.327	0.713	0.737
Childrens' python	0.431	0.661	0.443	—	0.379	0.200	0.562	0.590
Huntsman spider	0.554	0.762	0.458	0.621	—	0.291	0.678	0.703
Funnelweb spider	0.752	0.887	0.673	0.800	0.709	—	0.837	0.853
Centipede	0.372	0.603	0.287	0.438	0.322	0.163	—	0.525
Odorless control	0.344	0.575	0.263	0.410	0.297	0.147	0.475	—

DISCUSSION

Our results indicate that adults and juveniles respond differently to the chemical cues of a variety of predators and nonpredators. Adults showed no preferences between predators and nonpredators, indicating that either they could not detect predator odors or they did not avoid detected odors because they were no longer a threat. Juveniles, on the other hand, avoided all but one of the predator treatments in comparison to the control. This is the first study to show a reptile antipredator response to invertebrate predator odors. Specifically, juvenile *E. heatwolei* avoided the funnelweb spider over all other treatments.

Our knowledge of the specific chemicals that mediate antipredator responses in lizards is poor. However, evidence suggests that these chemicals are most likely lipids that originate from the integument of the predator (Dial, 1990). Integumentary lipids are thought to play an important role in communication among predator conspecifics (Simon, 1983). These substances also may be deposited on the substrate, and they have low volatility, so it is not unrealistic to expect that prey species may be able to detect these chemicals later in the absence of the predator (Schwenk, 1995).

Chemical Detection of Predators. Chemoreception is believed to be an important mechanism for detecting the presence of predators when other cues are

not available, such as in nocturnal species like geckos (Dial et al., 1989; Dial and Schwenk, 1996), or in low-visibility habitats, such as for tadpoles in turbid water (Schley and Griffiths, 1998). Low-visibility habitats also may include structurally complex habitats where prey may approach predators or be approached by them without detecting them visually. In such environments, more mobile animals such as active foragers may encounter predators at a greater rate (Anholt et al., 1996). *Eulamprus heatwolei* is an active forager that inhabits a complex habitat and uses chemoreception in social interactions. Thus, we expected that *E. heatwolei* would use chemoreception to avoid retreat sites treated with predator odors.

Adult *E. heatwolei* did not respond to red-bellied black snake odors. This result may seem surprising, as this snake species is sympatric with the lizards and is known to prey upon *E. heatwolei*. However, the ability of an individual to detect a predator is not dependent solely on aspects of the prey's ecology, but also on the behavior and ecology of the predator. For chemical cues to be useful in assessing predation risk, they must provide reliable information about the presence and identity of a predator. For example, the American toad, *Bufo americanus*, showed no response to odors of a known predator, the garter snake, *Thamnophis sirtalis* (Heinen, 1994). A lack of response may be because the substrate is likely to be permeated with the predator odors regardless of whether it is in the immediate vicinity; thus, chemicals do not provide a reliable cue. However, *Bufo cognatus* and *Bufo microscaphus* were both able to detect snake predator odors (Flowers and Graves, 1997). In this example, predator odor was a reliable cue because the snakes revisited the same foraging area daily. Chemical cues are most likely to be useful in detecting slow-moving predators that are confined to certain areas, such as ambush foragers (e.g., Gelowitz et al., 1993). Chemical information about fast moving and wide-ranging predators such as the red-bellied black snake may not be as reliable, because the chemicals will probably linger long after the snake has gone. Responding to the odors of predators that have left the area would incur unnecessary costs (Kats and Dill, 1998).

The only predator in our study that is naturally confined to a small area is the funnelweb spider, which waits at the entrance of its burrow for prey to come close. Because of its small size, the funnelweb is not a threat to adult *E. heatwolei* (in fact, observations suggest that adults may prey on funnelweb spiders). However, they do pose a predation threat to juveniles (Daniels and Heatwole, 1984). Retreat sites treated with the odor of funnelweb spiders were avoided by juvenile *E. heatwolei* more than any other scent, suggesting that chemical cues are a reliable means of detecting the risk of predation by this spider.

It is known that phylogeny can influence the distribution of chemical discrimination of prey, but there also is evidence that foraging mode affects the use of prey chemical discrimination independent of phylogeny (Cooper, 1994a). Changes in prey chemical discrimination are linked to changes in foraging mode, and this may show that prey chemical discrimination is adaptively matched to foraging mode

(Cooper, 1994b). Is chemical detection of predators also influenced by foraging mode? Published data suggest that it is. Like prey detection, the detection of predators by chemosensory means has been reported only in active foragers (however, no studies of chemical predator detection in iguanian ambush foragers have been reported) [e.g., eublepharids (Dial et al., 1989); lacertids (Thoen et al., 1986)].

A number of reasons could explain why this could be the case. First, lizards may simply be taking advantage of an already well-developed sense and applying it to other aspects of their ecology. Second, lizards that are active foragers may have other aspects of their ecology in common that influence the use of chemical predator detection. Third, foraging mode may have direct implications on the use of chemical signals for predator detection. Finally, any combination of the above may be true. Iguanian lizards may have little to gain from detecting predator odors because they tend to be sedentary species that escape predators by fleeing to known shelters rather than leaving the area. Furthermore, major predators of ambush foragers are likely to be active foragers (Cooper, 1994a). Detection of active foragers via chemical cues may not be as useful as the detection of ambush predators because odors are persistent and do not reliably indicate the location of fast-moving or wide-ranging predators. Active foraging lizards, on the other hand, are likely to encounter ambush predators, and chemical cues are likely to be reliable for detecting these predators because they are sedentary.

Our results for *E. heatwolei* support the idea that chemical detection of predators is not solely dependent on foraging mode. Adults of this actively foraging lizard species did not distinguish between the odor of predators and the control. This result is probably not a response to the inability to detect predators, but because the predator odors presented were derived from active foragers. This indicates that the absence or presence of chemical detection of predators is not the result of the lizards foraging mode directly, but that chemical cues are more useful in detecting ambush predators than active foragers and that ambush predators are more likely to pose a predation threat to active foragers. The fact that juvenile *E. heatwolei* responded most strongly to the odor of an ambush forager also supports this idea.

It has been suggested here and elsewhere (Downes and Shine, 1998) that chemical detection of predators is, in part, dependent on the ecology of the predator in question. While this idea makes intuitive sense, it remains to be demonstrated. Our study supports this idea; however, it is necessary to conduct experiments that explicitly test this hypothesis. Testing prey with the odors of numerous predators that pose an equal predation threat but differ in aspects of their ecology, such as foraging mode, may provide valuable information on this problem.

Another aspect of chemical predator detection that has been neglected is whether chemical cues are used by prey that themselves are ambush foragers. This has been looked at in the use of chemicals for foraging but not for predator

detection. Addressing this issue is pivotal to our understanding of the evolution of chemical communication. It is necessary that even negative results be published in this area if we are to gain an accurate picture of the use of chemical signals and the selection pressures that act on this mode of communication.

Age-Specific Changes in Predator Avoidance Behavior. Although not responding to predator cues appropriately can be lethal, an excessive or unnecessary response also can have detrimental effects for prey (Beldon et al., 2000). Similarly, if predators are not uniformly risky to prey, prey may respond more strongly to predators that pose a greater threat (Kats and Dill, 1998). For example, many amphibians respond only to predators that have consumed conspecifics (e.g., Wilson and Lefcort, 1993). Other evidence suggests that individuals may differ in their response to predators. For example, prey populations that experience lower predation pressures in response to different predators or predator densities respond less vigorously to predator odors (e.g., Ducey and Brodie, 1991; Mathis et al., 1993). Differential responses also may occur within populations because of changing predation pressures on different life stages. Such a phenomenon may lead to a change in antipredator responses during ontogeny (e.g., Wahle, 1992; Anholt et al., 1996). The more specific the information on potential risk, the more useful it may be in behavioral decision making.

Juvenile *E. heatwolei* avoided predator odors more than adults. Similar results have been demonstrated for other species. Some garter snakes show ontogenetic shifts in antipredator behavior in response to visual predator stimuli (Herzog et al., 1992). In lobsters, small individuals increased shelter use in response to predator odors, whereas large lobsters responded to these odors with aggressive displays (Wahle, 1992). In the common lizard (*Lacerta vivipara*), juveniles refrained from basking in the presence of predator odors, whereas adult basking behavior remained the same as in control tests (Van Damme et al., 1995). The study by Van Damme et al. (1995) differs from ours, however, in that juveniles in their study were inexperienced hatchlings and were compared to adults from the field. In our study, juveniles were caught in the field at the same time and from the same location as adults and so, presumably, would have been exposed to similar predators. Thus, previous experience is less likely to be a factor determining adult and juvenile response.

A change in antipredator response with individual size is not surprising because smaller individuals often are more vulnerable to predation than larger individuals (McCormick and Polis, 1982). This difference in predator threat is not only because more predators can eat smaller individuals, but also because a number of important antipredator mechanisms are associated with age and size. For instance, smaller individuals may not have developed a full array of defensive mechanisms present in larger individuals. Such is the case in some tadpoles, which become toxic as they increase is size (Anholt et al., 1996). In reptiles, relatively small individuals also may be less able to defend themselves because of lower stamina associated with anaerobic and aerobic capacity (Pough, 1977, 1978) and be less

able to run away from predators because size is often positively correlated with speed (Garland, 1985). Younger individuals also may be less familiar with their environment (Burger, 1990).

In conclusion, the ability of lizards to detect predators via chemical cues is not only dependent on their own ecology but also on the ecology of the predator in question. Predators must pose a sufficient threat and cues must be reliable for chemosensory mechanisms of predator detection to be an advantage. This holds true not only for different prey species or populations, but also for different life stages within a species.

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BROOK CHAR (*Salvelinus fontinalis*) CAN DIFFERENTIATE CHEMICAL ALARM CUES PRODUCED BY DIFFERENT AGE/SIZE CLASSES OF CONSPECIFICS

REEHAN S. MIRZA* and DOUGLAS P. CHIVERS

Department of Biology, 112 Science Place
University of Saskatchewan
Saskatoon, SK S7N 5E2, Canada

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Abstract—A wide diversity of aquatic organisms release chemical alarm cues when captured by a predator. For most animals, it is not known whether the specific chemicals that comprise the alarm cue are conserved as prey animals age. In this study, we tested whether brook char (*Salvelinus fontinalis*) can differentiate alarm cues produced by individuals of different ages/sizes. In separate laboratory experiments we exposed small brook char and large brook char to chemical alarm cues from small brook char, large brook char, and a control of swordtails (*Xiphophorus helleri*). Both small and large brook char responded with antipredator behavior to chemical alarm cues from both small and large char, but not to those from swordtails. Small char responded with a greater response intensity to cues of small char than to cues of large char. In contrast, large char responded with a greater response intensity to cues of large char than to cues of small char. These results suggest that chemical(s) that act as the alarm cue for fish of different age/size classes may be: (1) identical and that there may be other chemical(s) that allow the test fish to distinguish between cues from fish of different ages/sizes, or (2) the cues are not identical, but similar enough to be recognized.

Key Words—Brook char, alarm cue, ontogenetic shift, antipredator behavior, Salmonidae.

INTRODUCTION

A wide diversity of aquatic organisms release chemical alarm cues when captured by a predator (reviewed in Chivers and Smith, 1998). Chemical alarm cues serve to warn nearby individuals of potential threat in the vicinity. Individuals warned by

* To whom correspondence should be addressed. E-mail: mirza@skyway.usask.ca

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alarm cues may gain a survival benefit by avoiding or evading the predator during an encounter (Mathis and Smith, 1993; Wisenden et al., 1999; Mirza and Chivers, 2001a).

The importance of chemical cues may be heightened in situations where visual cues are limited, such as at night, in highly structured habitats, or in areas of high turbidity (Smith, 1992). Furthermore, chemical cues function well in aquatic media because a large number of compounds can dissolve in water, giving a large number of potential chemical cues to be detected (Kleerekoper, 1969; Hara, 1994). Although controversy exists concerning the function of chemical alarm cues (Magurran et al., 1996; Smith, 1997; Brown and Godin, 1999), there exists considerable evidence for the antipredator function of alarm cues for both signal senders and receivers (Smith, 1992; Chivers and Smith, 1998). Thus, chemical cues provide reliable information to assess predation risk (Kats and Dill, 1998; Wisenden, 2000).

The use of alarm cues to assess predation risk may change as animals age. Many organisms undergo ontogenetic shifts in diet and/or habitat during their life cycle (Werner and Gilliam, 1984). For example, many fishes commonly undergo an ontogenetic shift from feeding predominantly on invertebrates to piscivory (Werner and Gilliam, 1984; Olsen, 1996; Brown et al., 2001). In regard to chemical alarm cues, we do not know if the chemical(s) that comprise the cue are conserved with age. Moreover, we do not know if prey animals respond to the same chemical alarm cue with the same intensity at different stages in the life cycle.

Previous studies have demonstrated that prey animals respond with antipredator behavior to chemical alarm cues from similar age/size conspecifics (reviewed in Chivers and Smith, 1998; Summey and Mathis, 1998). However, little work has examined whether chemical alarm cues are conserved with age/developmental stage. Belden et al. (2000) found that juvenile western toads (*Bufo boreas*) recognize and avoid areas labeled with chemical cues from snakes (*Thamnophis sirtalis concinnus*) fed juvenile conspecifics, but not from snakes fed conspecific tadpoles.

We examined whether small and large brook char (*Salvelinus fontinalis*) could differentiate alarm cues produced by individuals of different size/age. We exposed small and large char to chemical alarm cues from injured small char, injured large char, and injured swordtails (*Xiphophorus helleri*). Swordtails represent a generalized injured fish cue to which char do not respond with antipredator behavior (Mirza and Chivers, 2000, Mirza and Chivers, 2001a,b). Small char were $\sim$5 months old whereas large char were 1 year older. We employed fish of a different age/size, but all individuals were juveniles. Brook char typically reach sexual maturity at age 3 and have a life-span that ranges from 5 to 8 years (Scott and Crossman, 1973). If the chemical(s) that comprise the cues are conserved with age, we predict that both small and large char should respond to injured cues from each other with the same intensity. If the chemicals are not conserved, then

responses should be age specific (small char should respond to small char, but not large char and vice versa).

METHODS AND MATERIALS

Fish Maintenance. We obtained one group of juvenile brook char from the provincial hatchery in Ft. Qu'Appelle, Saskatchewan, Canada, in May 2000 and a second group of juvenile char in May 2001. The two groups were kept in separate 350-liter artificial stream tanks at 14°C on a 14L:10D photoperiod. Fresh dechlorinated water was introduced into each holding tank at a rate of 1 liter/min. Char were fed daily on a combination of commercial trout pellets and frozen brine shrimp, *Artemia* spp.

Preparation of Stimulus. Skin extracts were prepared from 10 small brook char (mean ± SD, 4.30 ± 0.31 cm standard length), 2 large brook char (7.6 and 8.2 cm), and 8 swordtails (4.91 ± 0.32 cm). Fish were humanely killed with a single blow to the head in accordance to guidelines set by the Canadian Council on Animal Care. A fillet of skin was removed from both sides of each fish and placed in 100 ml of chilled glass-distilled water. Approximately 25 cm^2 of skin was collected from each of the three groups. Skin was homogenized, filtered through filter floss to remove the larger particles, and then diluted in distilled water to make a final volume of 400 ml. Each char was exposed to the equivalent of 1 cm^2 of skin. Lawrence and Smith (1989) report that for fathead minnows (*Pimephales promelas*) 1 cm^2 of minnow skin provides an active space of 58,000 liter, which is equivalent to a cube 3.9 m on a side. The active space created by 1 cm^2 of char skin is unknown. Stimuli were pipetted into sample bags in 25-ml aliquots and frozen at –20°C until used.

Response of Small Char to Conspecific Alarm Cues. We exposed individual small brook char (mean ± SD, 4.86 ± 0.09 cm, $N = 19$) to three treatments: (1) skin extract from small brook char, (2) skin extract from large brook char, and (3) skin extract from swordtails. Each char received each of the three treatments, and the presentation of the stimuli was randomized. There was a 24-hr period between each successive treatment for each char.

Each test chamber was set up as a gravity flow-through system (modified from Petranka et al., 1987). Water flowed into one of two 60-liter header tanks, then spilled over into a 113-liter test chamber (measuring 54 × 44 × 36 cm), and finally down the drain at a rate of 1 liter/min. A single plastic tube ran into one of the two header tanks for introduction of chemical stimuli. Each test chamber was subdivided into two compartments by an opaque barrier with screen mesh at either end. The barrier was 12 cm from the long wall of the tank. This created an observation area of 54 × 32 × 36 cm. Behind the barrier, two small powerheads were used to create a circular flow in the tank. Each test chamber contained a single

centrally located shelter object constructed from a ceramic tile (20 × 10 cm) with three glass legs (7 cm). A single plastic tube was situated at the right hand side of the chamber for introduction of food. A grid of 9- × 9-cm squares was constructed on the front of the barrier to quantify movement of fish in the tank (Figure 1).

Fish were allowed to acclimate in the test chambers for 24 hr prior to trials. Each trial was 18 min in length and consisted of an 8-min pre- and 8-min poststimulus period, with a 2-min stimulus introduction period in between. Dye trials indicated that it took approximately 1.5 min for the stimulus to enter the test chamber from the header tank. At the beginning of both the pre- and poststimulus periods, approximately 5 ml of previously frozen brine shrimp was introduced into the tank. The food was added to stimulate activity and remained in the water column for only a few minutes. Char were observed to forage predominantly in the water column, rather than pick food off the bottom. The water flow was switched over to the second header tank after the prestimulus period to allow introduction of the chemical stimulus. Tanks were drained and cleaned out between successive trials. Test fish were held in the test tanks until exposed to all three treatments.

During both the pre- and poststimulus periods, we recorded the number of line crosses and the number of feeding bites. We also recorded occurrence of motionlessness (stationary for a minimum of 30 sec on either the bottom or in the water column). A line cross was recorded when 75% of the fish's body crossed from one grid square to another. We measured the length of each fish and converted the number of line crosses into number of body lengths moved. This was done in order to allow comparison of movement between fish of different sizes. Changes between the prestimulus and poststimulus periods for each response variable were calculated, and the difference in changes between pairs of treatments was analyzed by using Wilcoxon sign rank tests (Siegel and Castellan, 1988). The family-wise error rate was assessed and controlled by using the modified Bonferroni test (Keppel, 1982). The modified Bonferroni test specifies that corrections to the family-wise error rate be introduced only when the number of comparisons exceeds $k - 1$, where k is the number of treatments (Keppel, 1982). The comparisons must be based on a priori predictions. In this experiment, there were three treatments; thus, the family-wise error rate was held to $P = 0.033$. Occurrence of motionlessness was compared with a Cochran Q test (Siegel and Castellan, 1988). Based on predictions of reduced activity and reduced foraging of brook char to conspecific alarm cues found in our previous studies (Mirza and Chivers, 2000, 2001a,b), we conducted one-tailed tests.

Response of Large Char to Conspecific Alarm Cues. In this experiment, we exposed large brook char (9.90 ± 0.36 cm, $N = 19$) to chemical alarm cues from injured large char, injured small char, and injured swordtails. Each char was tested individually and received each of the three treatments in a randomized order. The experimental setup, procedure, and analysis were identical to that used in experiment 1.

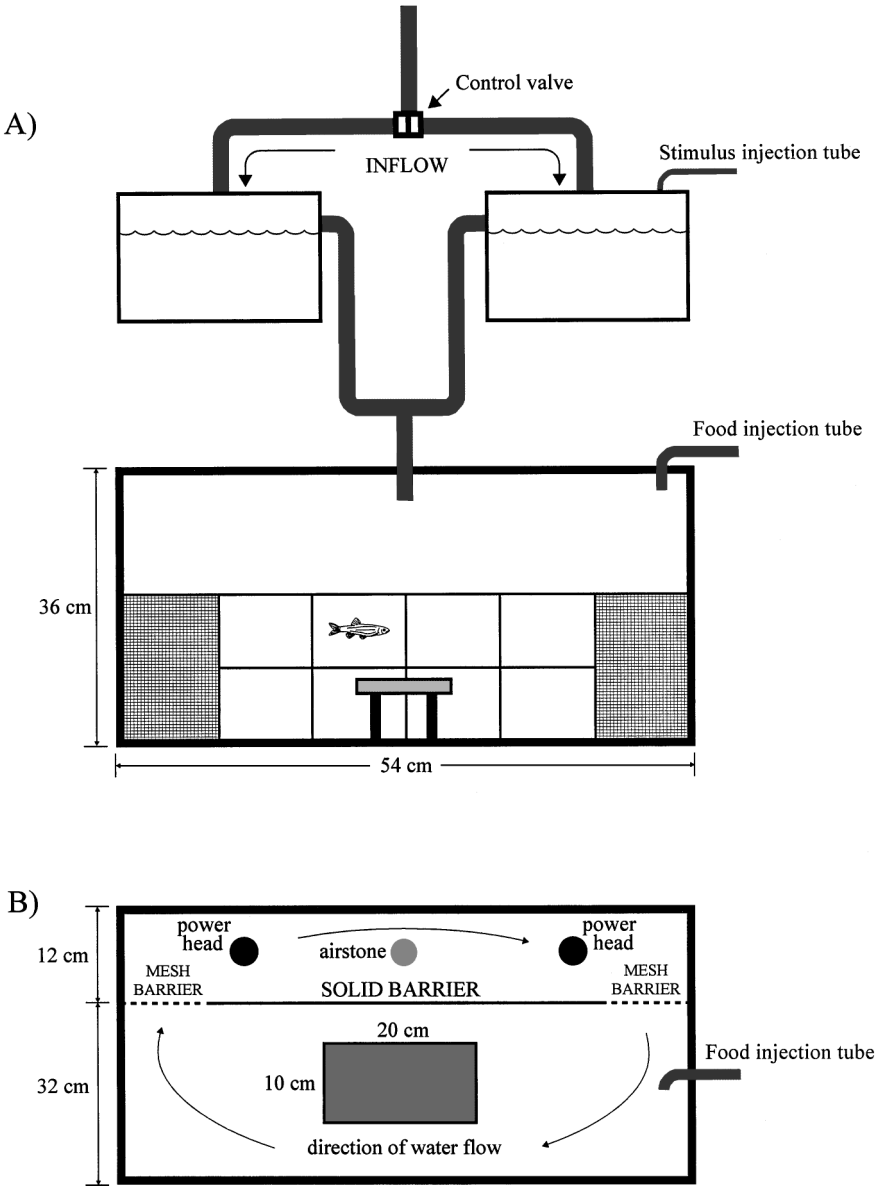


FIG. 1. Schematic diagram representing the gravitational flow-through apparatus used in experiment 1 illustrating front (A) and top (B) views.

RESULTS

Response of Small Char to Conspecific Alarm Cues. Results of the Wilcoxon sign rank tests revealed that small char reduced their activity (number of body lengths moved) in response to small char cues ($Z = -3.42$; $P < 0.001$) and to large char cues ($Z = -2.86$; $P = 0.002$) when compared to swordtail cues. Moreover, small char showed a greater reduction in movement in response to cues from small char than to cues from large char ($Z = -2.03$; $P = 0.021$; Figure 2).

Small char reduced their number of feeding strikes when exposed to small char skin extract and large char skin extract compared to exposure to swordtail

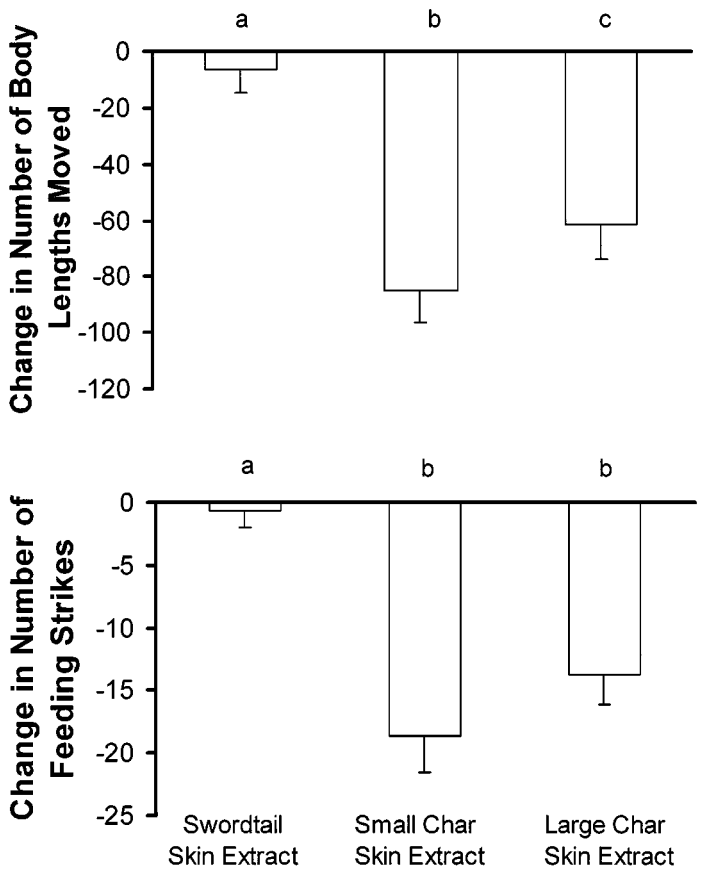


FIG. 2. Mean (+SE) of changes in number of body lengths moved (top) and number of feeding strikes (bottom) for small brook char exposed to skin extract from small brook char, large brook char, and swordtails. Different letters over bars denote significant differences at $P < 0.05$ (see text for details).

skin extract ($Z = -3.52$, $P < 0.001$; $Z = -3.29$, $P < 0.001$). There was a non-statistically significant trend for small char to reduce foraging strikes in response to cues of small char than to cues of large char ($Z = -1.83$, $P = 0.034$; Figure 2).

Small char were motionless in 9 of 19 trials when exposed to small char skin compared to 6 of 19 trials when exposed to large char skin and 1 of 19 trials when exposed to swordtail skin (Cochran's $Q = 9.8$, $P = 0.004$).

Response of Large Char to Conspecific Alarm Cues. When compared to responses to the control swordtail cues, large char reduced their activity in response to both cues of large char ($Z = -2.98$; $P < 0.002$) and to cues of small char ($Z = -2.33$; $P = 0.010$). Moreover, large char reduced activity to a greater extent in response to large char cues than to small char cues ($Z = -1.87$; $P = 0.031$; Figure 3).

Char reduced their number of feeding strikes when exposed to large char cues and small char skin cues when compared to exposure to swordtail cues ($Z = -2.63$, $P = 0.005$; $Z = -2.02$, $P = 0.014$). However, there was no significant difference in the level of reduction in foraging activity for large char exposed to large char cues compared to exposure to cues of small char ($Z = -0.78$, $P = 0.218$; Figure 2).

Large char were motionless in 8 of 19 trials when exposed to large char skin extract compared to 3 of 19 trials when exposed to small char skin extract and 2 of 19 trials when exposed to swordtail skin extract (Cochran's $Q = 7.75$, $P = 0.011$).

DISCUSSION

Our results indicate that brook char are capable of distinguishing between alarm cues produced by individuals of different ages/sizes. Small brook char responded to conspecific alarm cues produced by both small and large individuals. However, there was a significantly greater reduction in activity when char were exposed to alarm cues from conspecifics of the same age/size than from older/larger individuals. Conversely, older/larger char reduced activity significantly more in response to alarm cues from large char than small char. Given that predators are often specific in the size of prey that they consume, it is adaptive for prey to respond most strongly to alarm cues of individuals that are the same size as the sender.

Ours is one of the first studies to examine how responses to alarm cues change through ontogeny. In another study, Belden et al. (2000) examined whether conspecific alarm cues were conserved in amphibians that underwent metamorphosis. They exposed juvenile western toads to chemical cues of red-spotted garter snakes fed either juvenile conspecifics, snakes fed larval conspecifics (tadpoles), water conditioned by juvenile toads, or water conditioned by earthworms. Juvenile toads avoided areas labeled with chemical cues from snakes fed juvenile conspecifics, but not to chemical cues from snakes fed tadpoles or the other two treatments. Previous work had shown that western toad tadpoles respond to alarm

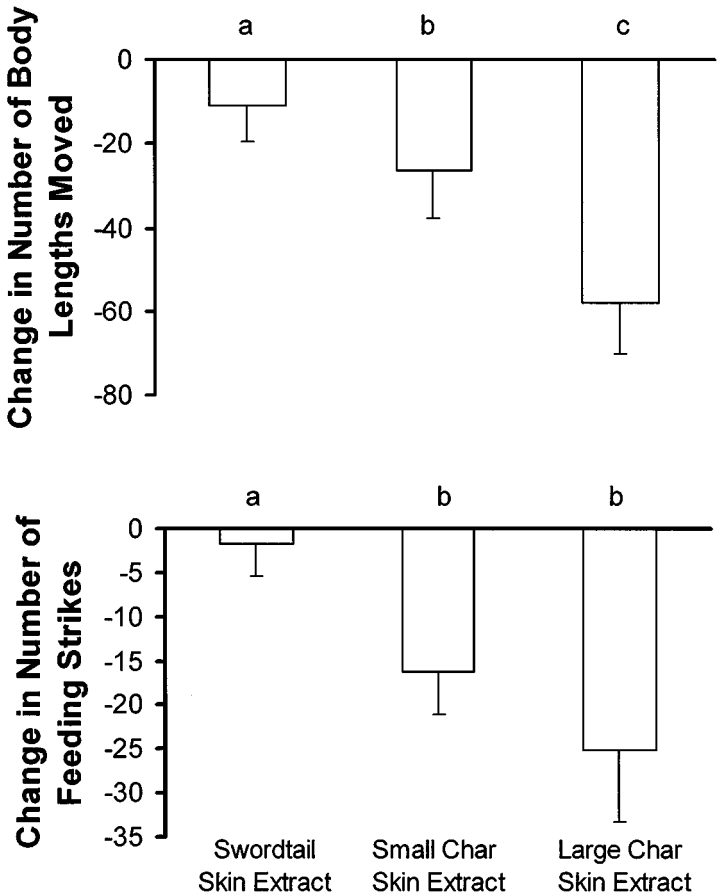


FIG. 3. Mean (+SE) of changes in number of body lengths moved (top) and number of feeding strikes (bottom) for large brook char exposed to skin extract from small brook char, large brook char, and swordtails. Different letters over bars denote significant differences at $P < 0.05$ (see text for details).

cues from conspecific tadpoles (Hews and Blaustein, 1985; Hews, 1988; Kiesecker et al., 1996). Belden et al. (2000) suggest that the lack of response to snake-fed-tadpole cues may be the result of the lack of conservation of the alarm cue between larval and juvenile forms, differential processing of larval and juvenile alarm cues in the gut of the predator, or an inability of juveniles to detect the chemical cue due to changes in chemoreception attributed to metamorphosis. Alternatively, a lack of response may be due to larval and juvenile toads not being members of the same prey guild.

Several studies have documented that salmonid fishes (salmon, trout, and char) use chemical alarm cues to assess their level of predation risk. (Brown and Smith, 1997; Berejikian et al., 1999; Mirza and Chivers, 2000, 2001a,b). However, little is known about the chemical nature of salmonid alarm cues. In one study, Mirza and Chivers (2001b) demonstrated cross-species reactions to chemical alarm cues among three different species of salmonids (rainbow trout, *Oncorhyncus mykiss*; brown trout, *Salmo trutta*; and brook char). For all of the species, the responses to cues of conspecifics were of greater intensity than to cues of heterospecifics. Mirza and Chivers (2001b) concluded that the chemical(s) that act as the alarm cue may be: (1) identical and that there may be chemical(s) that allow the fish to distinguish between conspecifics and heterospecifics, or (2) that the chemical(s) that act as the cue are not identical but are similar enough to be recognized.

In this study, we found that both small and large char responded to alarm cues produced by each other, but that the intensity of the response was lower for different sized individuals. Consequently, these results indicate that the chemical(s) that act as the alarm cue for fish of different age/size classes may be similar but not identical. Alternatively, the chemical(s) may be identical, and there may be other chemicals present that allow the test fish to distinguish among individuals of different ages/sizes. The level of complexity and specificity of chemical alarm cues among salmonid fishes appears to be sophisticated and requires further study.

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VOLATILE PRODUCTION BY BUDS AND COROLLAS OF TWO SYMPATRIC, CONFAMILIAL PLANTS, *Ipomopsis aggregata* AND *Polemonium foliosissimum*

REBECCA E. IRWIN[1,2,*] and BOB DORSETT[2,3]

[1]*Insitute of Ecology*
University of Georgia
Athens, Georgia 30602

[2]*Rocky Mountain Biological Laboratory*
Crested Butte, Colorado 81224

[3]*Natural Science Department*
Colorado Northwestern Community College
Meeker, Colorado 81641

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Abstract—We used solid-phase microextraction of headspace samples followed by gas chromatography and mass spectrometry to identify and quantify the major volatile compounds produced by the buds and corollas of *Ipomopsis aggregata* (Polemoniaceae), and we compared them to a sympatric, confamilial plant species, *Polemonium foliosissimum*. The two species have distinct floral morphologies and pollinators, but share a common predispersal seed predator that oviposits under the calyces of buds. *Ipomopsis aggregata* buds emitted fewer compounds than the corollas. The buds of *I. aggregata* were dominated by α-pinene and, to a lesser extent, β-pinene. The corollas of *I. aggregata* emitted a mixture of 10 compounds. Like the buds, *I. aggregata* corollas produced relatively high concentrations of α-pinene as well as caryophyllene. In addition, the emission of four terpenoids, three esters, and one ketone added to the volatile bouquet of the corollas. *Polemonium foliosissimum* buds also emitted fewer compounds than the corollas. The buds of *P. foliosissimum* were dominated by β-pinene and, to a lesser extent, α-pinene. The corollas of *P. foliosissimum* were also dominated by β-pinene along with three terpenoids, three alcohols, one ester, and two aldehydes. Comparing *I. aggregata* and *P. foliosissimum*, bud samples from both species produced considerable amounts of α- and β-pinene. *Ipomopsis aggregata* emitted more α-pinene than β-pinene, while *P. foliosissimum* emitted more β-pinene. The corollas of the two species, however, differed in their volatile bouquet. The partition of volatiles between *I. aggregata* and *P. foliosissimum* buds and corollas and differences in volatile

*To whom correspondence should be addressed. E-mail: rirwin@arches.uga.edu

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production between *I. aggregata* and *P. foliosissimum* are consistent with selection pressures exerted by organisms interacting with these plants.

Key Words—Floral volatiles, *Ipomopsis aggregata*, Polemoniaceae, *Polemonium foliosissimum*, pollinators, seed predators.

INTRODUCTION

Floral and vegetative fragrances mediate insect identification of plants (Williams, 1983; Robacker et al., 1988; Harborne, 1993). Volatile odors guide pollinators to nectar and pollen rewards or mating sites (e.g., Dodson et al., 1969; Borg-Karlson et al., 1985; Heath et al., 1992; Dobson et al., 1996; Raguso et al., 1996; Roy and Raguso, 1997), advertise vegetative or floral food for herbivores (e.g., Liu et al., 1988; Hopkins and Young, 1990), and serve as oviposition cues for insects (e.g., Dorsett and Pike, 1995; Jallow et al., 1999). Intra- and interspecific variation in volatiles help insects choose among individual plants of the same species (Pellmyr, 1986; Groth et al., 1987; Tollsten and Bergström, 1989) and among individual plants of different species (Dodson, 1970; Williams and Dodson, 1972; Knudsen et al., 1993; Dobson et al., 1996). Yet, floral odors have received little attention compared to studies that examine floral color, size, and shape as cues for visiting insects (for reviews see Faegri and van der Pijl, 1979; Jones and Little, 1983; Proctor et al., 1996). The paucity of studies on volatile odors as attractants may be due, in part, to the difficulty in identifying and quantifying odors produced by plants (for a review see Raguso and Pellmyr, 1998). Nonetheless, insects often use variation in floral odor at both long and short distance intervals to choose plants and may exert phenotypic selection on floral scent production. Here, we explore differential volatile production within and between two plant species in relation to known insect visitors.

Limited work has shown that the volatile odors emitted by plants vary at several levels, including variation among floral parts within individuals (e.g., Meeuse et al., 1984; Dobson et al., 1990, 1996), among individuals within species (e.g., Galen and Kevan, 1980; Chang et al., 1988; Galen and Newport, 1988), and among species (e.g., Knudsen et al., 1993; Dobson et al., 1996). For example, flower parts, such as petals, sepals, anthers, pollen, and/or nectar, often differ in fragrance chemistry and intensity and may signal to insects the location of food or oviposition sites within plants (e.g., Dobson et al., 1990, 1996, 1999). Scent polymorphisms within species may attract different suites of pollinators, resulting in assortative mating based on scent preference (e.g., Galen and Kevan, 1980; Galen and Newport, 1988). In addition, scent variation among closely related species may result in reproductive isolation (e.g., Dodson, 1970; Williams and Dodson, 1972).

Despite the documented variation in floral-scent chemistry, similarities in floral scents among closely related plants species are striking. Closely related plants

often produce similar suites of chemical compounds, although the distribution of compounds does not necessarily follow phylogenetic branches (e.g., Becerra, 1997). The macroevolutionary importance of scent (or chemical) production may be critical to understanding the evolution of host shifts by generalist pollinators and phytophagous insects and to understanding the evolution of plant–insect interactions in general (Ehrlich and Raven, 1964).

Plants in the family Polemoniaceae provide a unique opportunity to test the importance of differential volatile production in attracting various insect visitors. The Polemoniaceae include a diversity of vegetative and floral forms with a wide-ranging assemblage of pollinators, nectar robbers, herbivores, and seed predators (for a review see Grant and Grant, 1965). A wealth of studies have investigated how interacting organisms respond to variation in vegetative and floral diversity in the family (e.g., Brody, 1992; Galen, 1983, 1996, 1999; Brody and Mitchell, 1997; Campbell et al., 1997); however, the importance of volatile odors has been virtually ignored (but see Galen and Kevan, 1980; Galen and Newport; 1988; Dorsett and Pike, 1995). Here, we examine intraspecific and interspecific variation in volatile-odor production of the buds and corollas of two members of the Polemoniaceae, *Ipomopsis aggregata* and *Polemonium foliosissimum*.

The plant and floral morphologies of *I. aggregata* and *P. foliosissimum* are distinct. *Ipomopsis aggregata* produce a single stalk with numerous red, trumpet-shaped flowers that are pollinated primarily by hummingbirds (*Selasphorus platycercus* and *S. rufus*) (Waser, 1978) and are nectar robbed by bumblebees (*Bombus occidentalis*) (Irwin and Brody, 1998, 1999). To the human nose, *I. aggregata* floral corollas range in smell from sweet (like applesauce) to unscented, and the buds often smell pungent. In contrast, *P. foliosissimum* produce multiple stalks with purple, campanulate flowers that are pollinated primarily by bumblebees (*Bombus* spp.) (Zimmerman, 1980). The petals of *P. foliosissimum* flowers usually smell sweet (like sweet clover), and the buds smell pungent or skunky (Dorsett and Pike, 1995). Despite the visual dissimilarity of the two species, they share a common predispersal seed predator, *Hylemya* sp. (Anthomyiidae) (Zimmerman et al., 1984). *Hylemya* lays its eggs under the sepals of buds on both species. The eggs hatch after approximately 14 days, and the larvae burrow into the developing fruits and eat the seeds before they disperse. *Ipomopsis aggregata* and *P. foliosissimum* are the sole hosts for *Hylemya*. We hypothesized that because *I. aggregata* and *P. foliosissimum* share this seed predator, their buds may produce similar volatiles as attractants. However, because the species differ in their primary pollinators, we hypothesized that the corollas may produce distinct odors.

Our objective was to identify, quantify, and compare the volatile odors produced by *I. aggregata* and *P. foliosissimum*. We collected buds (plus gynoecium) and corollas (plus anthers) of *I. aggregata* and *P. foliosissimum* and identified and quantified their major volatiles using solid-phase microextraction (SPME)

followed by gas chromatography (GC) and mass spectrometry (MS). We addressed the following questions: (1) Do the buds and corollas of *I. aggregata* differ in the quality and quantity of volatile compounds produced? (2) Do the buds and corollas of *P. foliosissimum* differ in the quality and quantity of volatile compounds produced? (3) Do *I. aggregata* and *P. foliosissimum* differ in the quality and quantity of volatile compounds produced?

METHODS AND MATERIALS

Study System. We collected plant material from sympatrically growing populations of *I. aggregata* and *P. foliosissimum* around the Rocky Mountain Biological Laboratory (RMBL), Gunnison County, Colorado, USA. Collections were made in July and August 1999 during the peak blooming of both species. Additional collections were made in July 2001 to further identify unknown peaks.

SPME Sampling and Gas Chromatography of I. aggregata. We used the Supelco SPME portable field sampler to collect floral volatiles and then injected the SPME fiber directly into a gas chromatograph for separation and identification of compounds. The portable field sampler has a 75-μm-thick fiber coated with carboxen/polydimethylsiloxane as the stationary phase (no. 504831, Supelco, Sigma-Aldrich Co., Bellefonte, Pennsylvania). Fibers were conditioned initially for 30 min at 280°C, and after each GC injection, the fiber was desorbed at 250°C for 5 min in the GC injection port.

To analyze *I. aggregata* buds, we collected samples from six plants (12 buds per plant) and placed samples from the same plant into cleaned 4 ml glass headspace vials. To analyze *I. aggregata* corollas, we collected six 1 to 2-day-old corollas from each of nine plants into cleaned headspace vials. The vials were sealed with a predrilled PTFE septum. We collected all samples between approximately 0900 and 1100 at similar ambient conditions. In no case were samples pooled; therefore, we could assess plant-to-plant variation in volatile production. We realize that sampling excised tissues for volatiles could introduce physiological artifacts, such as interruption of the flow of metabolic precursors to the sites of volatile emission. However, to sample the volatiles emitted by *individual* floral parts, excision of tissues was essential, and it has been used in other studies identifying volatiles in individual floral parts (e.g., Dobson et al., 1996).

We allowed samples to equilibrate in the vials for approximately 15 min. The septum-piercing needle of the field sampler was then driven through the predrilled septum, and the fiber was injected into the sample vial. Care was taken to ensure that the fiber was extended fully but did not touch the plant tissue or sides of the vial. In all samples, we included 2 μl of 2,2,4-trimethylpentane or geraniol as internal standards (neither of these chemicals are emitted naturally by *I. aggregata* or *P. foliosissimum*). We adsorbed volatiles onto the fiber for approximately 30 min

at 27°C. The fiber was then retracted into the septum-piercing needle sleeve, withdrawn from the sample vial, and introduced into the GC.

We used an HP 402 gas chromatograph with a 30-m nonpolar SPB1 (polydimethylsiloxane) capillary column and flame ionization detector. Starting temperature was 40°C. Injector temperature was 200°C, and detector temperature was 250°C. We held the column temperature at 40°C for 4 min, ramped the temperature to 200°C at 10°C/min, then held the temperature constant at 200°C for 5 min. We did not ramp the temperature above 200°C because preliminary work found no active compounds above 200°C. We used helium as the carrier gas at 20 ml/min, with nitrogen makeup (40 ml/min). As controls, we included runs of the 2,2,4-trimethylpentane and geraniol standards to ensure that the chemicals were not adding extra peaks to the chromatographs. We also controlled for ambient contaminants with blank runs of SPME fibers adsorbed in clean, empty vials.

To correlate fragrance components with GC peaks, we first timed the arrival of various fragrances at the GC detector to the human nose. Then, to identify the chemicals produced by the floral parts, we ran standards of known volatiles obtained from Sigma Aldrich Inc., including α- and β-pinene, cineole, citronellol, coumarin, ethyl acetate, farnesol, geraniol, limonene, linalool, myrcene, and phenylethyl alcohol. We compared the retention times of the standard peaks to those in our floral samples. We confirmed uncertain peaks by augmentation and GC-MS.

SPME Sampling and Gas Chromatography of P. foliosissimum. We also used SPME to identify volatiles produced by *P. foliosissimum* buds and corollas. For buds, we collected samples of 12 buds per plant from each of 12 plants. For corollas, we collected samples from three plants, 10 corollas per plant. We collected 12 buds and 10 corollas per sample for *P. foliosissimum*, as opposed to 6 buds and 6 corollas for *I. aggregata*, because of differences in size between the buds and corollas of the two species. We did not include an internal standard in the corolla samples because we could not find a reliable standard that we also were sure was not covering any sample peaks. We used the same techniques for SPME, GC, and GC-MS as described above for *I. aggregata*.

Data Analyses. To test for differences in total volatile production by the buds and corollas of *I. aggregata*, we added the areas of all peaks from a sample and divided by the area of the standard peak for that sample. We then used ANOVA with floral structure (bud or corolla) as the independent variable and standardized total volatiles emitted (square-root transformed) as the response variable.

To compare relative quantities of the *individual* volatile compounds emitted by bud and corolla samples, we calculated the mean percent composition of each chemical emitted by the different structures. This was accomplished by dividing individual peak areas by the total area of all volatiles emitted on a chromatograph for each species. To compare these volatiles statistically, we standardized the GC peaks from *I. aggregata* buds and corollas and from *P. foliosissimum* buds by dividing the area of each peak on the chromatograph by the area of the standard peak

(2,2,4-trimethylpentane or geraniol). To test for differences in volatile emissions between *I. aggregata* buds and corollas, we used MANOVA with floral structure (bud or corolla) as an independent variable and standardized peak areas (square-root transformed) for each chemical detected as response variables.

In addition, to compare the volatiles emitted by *I. aggregata* and *P. foliosissimum* buds, we used MANOVA with plant species as the independent variable and standardized peak area (square-root transformed) for each chemical detected as response variables. Finding significant MANOVAs (see Results below), we then used univariate ANOVAs to determine which chemical emissions differed between *I. aggregata* buds and corollas and between *I. aggregata* and *P. foliosissimum* buds. Using MANOVA followed by univariate ANOVAs decreases the type I error rate (Rencher, 1995). Because we did not add an internal standard to the *P. foliosissimum* corolla samples, we could only compare qualitative differences between *P. foliosissimum* buds and corollas and between *I. aggregata* and *P. foliosissimum* corollas.

RESULTS

Ipomopsis aggregata Volatiles. The buds of *I. aggregata* emitted three volatile compounds, while the corollas emitted the same three compounds as the buds along with seven additional compounds (Table 1). The three compounds we detected from *I. aggregata* buds were the monoterpenes α-pinene (93.17%) and β-pinene

TABLE 1. COMPOSITION OF VOLATILES FROM *I. aggregata* BUDS AND COROLLAS COLLECTED BY SPME FOLLOWED BY GC

Compound	From buds (%, mean ± SE)[a]	From corollas (%, mean ± SE)[b]
1. Esters		
3-hexen-1-ol-acetate	0	9.46 ± 2.04
Butyl butanoate	0	0.94 ± 0.26
Hexenyl butanoate	0	2.12 ± 0.95
2. Monoterpenoids		
α-pinene	93.17 ± 1.30	23.23 ± 7.58
β-pinene	5.01 ± 0.94	2.25 ± 1.04
β-myrcene	0	8.69 ± 2.63
Unidentified 1	0	2.07 ± 0.59
3. Sesquiterpenoids		
Copaene	0	9.06 ± 2.25
Caryophyllene	1.82 ± 0.41	25.93 ± 5.12
4. Ketones		
2-methyl-6-methenyl-1,7-octodien-3-one	0	16.25 ± 4.81

[a] $N = 6$.
[b] $N = 9$.

(5.01%) and the sesquiterpene caryophyllene (1.82%). In total, the buds emitted approximately one half fewer volatiles than the corolla samples (bud versus corolla standardized mean $\pm$ 1 SE: 0.13 ± 0.10 vs. 0.28 ± 0.06); however, this difference was not statistically significant ($F_{1,13} = 3.77$, $P = 0.07$).

The dominant peaks from *I. aggregata* corollas were the terpenoids caryophyllene (25.93%) and α-pinene (23.23%) and the ketone 2-methyl-6-methenyl-1, 7-octodien-3-one (16.25%) (Table 1). Minor constituents in the corollas included three esters (4-hexen-1-ol-acetate, butyl butanoate, and hexenyl butanoate) and four terpenes (β-myrcene, β-pinene, copaene, and one unidentified monoterpene) (Table 1).

Analyses contrasting the relative emissions of the volatiles from the buds and corollas of *I. aggregata* showed that the buds and corollas emitted different floral fragrances (MANOVA: $F_{10,4} = 23.09$, $P = 0.0042$). This result is driven primarily by the buds emitting only three volatile compounds that we could detect (α- and β-pinene and caryophyllene), while the corollas emitted 10 compounds (dominated primarily by α-pinene and caryophyllene). We then compared the three compounds that the buds and corollas shared: α-pinene, β-pinene, and caryophyllene. The buds and corollas differed in the amounts of these compounds emitted (MANOVA: $F_{3,11} = 5.79$, $P = 0.013$). Specifically, the corollas emitted almost 100 times more caryophyllene than the buds (ANOVA: $F_{1,13} = 14.17$, $P = 0.0024$). However, the buds and corollas did not differ in the amount of α-pinene (ANOVA: $F_{1,13} = 0.57$, $P = 0.46$) or β-pinene (ANOVA: $F_{1,13} = 0.0308$, $P = 0.86$) emitted.

Polemonium foliosissimum *Volatiles.* From *P. foliosissimum* buds, we detected four compounds; the largest peaks included the monoterpenes α-pinene (25.91%) and β-pinene (60.50%). The volatile compounds 2-ethyl-1-hexanol (4.15%) and caryophyllene (9.44%) were minor constituents of the total area measured in the buds' GCs.

In the corolla samples, we detected the four compounds that were emitted from the buds along with six additional compounds (Table 2). As with the buds, the dominant peaks included the monoterpenes α-pinene (24.87%) and β-pinene (40.99%). In addition, the aldehyde benzeneacetaldehyde (9.88%) and the sesquiterpene caryophyllene (6.60%) added to the volatile bouquet. Minor constituents in the corolla samples included three alcohols (1-hexanol, 2-ethyl-1-hexanol, and phenylethyl alcohol), one sesquiterpenoid (copaene), one ester (benzeneacetic acid methyl ester), and one aldehyde (benzaldehyde) (Table 2).

Qualitative comparison of the bud and corolla samples showed that the two produced different relative amounts of volatile compounds (Table 2). The bud and corolla samples produced similar relative amounts of α-pinene (25.91% vs. 24.87%), 2-ethyl-1-hexanol (4.15% vs. 3.31%), and caryophyllene (9.44% vs. 6.60%). However, the buds emitted proportionally more β-pinene (60.79%) than the corollas (40.99%). Finally, six compounds were unique to the corolla samples, including two alcohols (1-hexanol and phenylethyl alcohol), one sesquiterpene

TABLE 2. COMPOSITION OF VOLATILES FROM *P. foliosissimum* BUDS AND COROLLAS COLLECTED BY SPME FOLLOWED BY GC

Compound	From buds (%, mean ± SE)[a]	From corollas (%, mean ± SE)[b]
1. Alcohols		
1-hexanol	0	2.46 ± 0.51
2-ethyl-1-hexanol	4.15 ± 0.84	3.31 ± 0.86
Phenylethyl alcohol	0	3.56 ± 1.92
2. Esters		
Benzeneacetic acid methyl ester	0	1.15 ± 0.35
3. Monoterpenoids		
α-pinene	25.91 ± 0.79	24.87 ± 5.53
β-pinene	60.50 ± 1.08	40.99 ± 2.28
4. Sesquiterpenoids		
Copaene	0	2.78 ± 2.07
Caryophyllene	9.44 ± 1.21	6.60 ± 4.29
5. Aldehydes		
Benzaldehyde	0	4.40 ± 3.11
Benzeneacetaldehyde	0	9.88 ± 7.43

[a] $N = 12$.
[b] $N = 3$.

(copaene), one ester (benzeneacetic acid methyl ester), and two aldehydes (benzaldehyde and benzeneacetaldehyde) (Table 2).

Comparison of Ipomopsis aggregata and Polemonium foliosissimum Volatiles. Ipomopsis aggregata and *P. foliosissimum* buds both emitted α-pinene, β-pinene, and caryophyllene, although in different amounts (MANOVA: $F_{3,14} = 4.88$, $P = 0.016$) (Tables 1 and 2). Relative to other compounds, *I. aggregata* buds emitted more α-pinene than *P. foliosissimum* ($F_{1,16} = 1124.32$, $P < 0.001$), whereas *P. foliosissimum* buds emitted more β-pinene ($F_{1,16} = 1067.53$, $P < 0.001$) and caryophyllene ($F_{1,16} = 24.21$, $P < 0.001$). In addition, the alcohol 2-ethyl-1-hexanol was unique to *P. foliosissimum* buds.

The corollas of *I. aggregata* and *P. foliosissimum* both emitted α- and β-pinene, although the *P. foliosissimum* corollas emitted proportionally more β-pinene than the *I. aggregata* corollas (Tables 1 and 2). In addition, both species also emitted copaene and caryophyllene, but *I. aggregata* corollas emitted proportionally more of both of these compounds than *P. foliosissimum* corollas (Tables 1 and 2). In addition, both species emitted six different compounds unique to each (Tables 1 and 2). In particular, *I. aggregata* may be distinguished by esters (butyl and hexenyl butanoate) and dominant amounts of caryophyllene and a ketone (2-methyl-6-methenyl-1,7-octodien-3-one) producing an overall applelike fragrance; while the corollas of *P. foliosissimum* may be distinguished by aldehydes, in particular, producing a sweet-clove fragrance.

DISCUSSION

Ipomopsis aggregata and *P. foliosissimum* buds and corollas emitted several similar compounds; however, the relative amounts of each compound differed between species and among flower parts. The buds of *I. aggregata* emitted the fewest compounds, dominated by α-pinene, β-pinene, and caryophyllene. The volatile bouquet from the corolla samples of *I. aggregata* was more complex than the buds, emitting 10 volatile compounds. Like the buds, volatiles from *I. aggregata* corollas were dominated by α-pinene and caryophyllene; however, the corollas were also characterized by unique ketones (2-methyl-6-methenyl-1,7-octodien-3-one), terpenoids (such as copaene and β-myrcene), and esters (3-hexen-1-ol-acetate, butyl butanoate, and hexenyl butanoate). In comparison, the buds of *P. foliosissimum* were dominated by β-pinene and, to a lesser extent, α-pinene, caryophyllene, and 2-ethyl-1-hexanol. The corollas of *P. foliosissimum* were also dominated by β-pinene along with a mixture of terpenoids (such as caryophyllene), alcohols, esters, and aldehydes. The dominance of β-pinene emitted by *P. foliosissimum* buds and corollas matches findings of Dorsett and Pike (1995). In addition, most of the volatiles emitted by *I. aggregata* and *P. foliosissimum* are also commonly found in a wide variety of angiosperm species (Knudsen et al., 1993).

Peak area in gas chromatography is sensitive to methodology, e.g., peak areas may vary depending on the adsorbents used in headspace collection and also the stationary phase in the chromatograph. Therefore, we cannot be certain that the peak areas collected by SPME quantitatively represent realized volatile production by the floral parts. However, similar peak patterns were obtained from *I. aggregata* and *P. foliosissimum* by using a dynamic headspace technique (flow-through air over a volatile trap containing Tenax GC and activated charcoal) (Dorsett and Pike, 1995; Dorsett and Irwin, unpublished data), and recent comparisons have shown that SPME is more sensitive than other headspace techniques to a wide range of volatile compounds, especially those at low concentrations, although SPME is less sensitive to other compounds, such as benzenoids (Faldt et al., 2000). Therefore, even though SPME fiber types in a static sampling environment may be somewhat limiting chemically, the fiber used here was probably adequate to detect the types and quantities of compounds emitted by *I. aggregata* and *P. foliosissimum*, especially since we were most interested in relative comparisons among floral structures and between plant species and not in absolute emission of volatiles.

The finding that all of the volatiles emitted by the buds of *I. aggregata* and of *P. foliosissimum* were also emitted by the corollas is not uncommon. Different floral parts on the same plant may emit the same compounds in variable quantities (e.g., Dobson et al., 1990, 1996). For example, in the plant *Papaver rhoeas* (Fabaceae), pollen odors differ from flower odors primarily in the proportions of volatiles produced (Dobson et al., 1996). In general, the biochemical pathways responsible for the production of volatile compounds in buds and corollas may be

similar, resulting in similar emissions of volatiles. Furthermore, insects visiting the plants may not distinguish among different scents emitted by different floral parts. Therefore, the bouquet of whole-flower, whole-plant, and even whole-patch scents may be important in attracting visiting insects and animals to the plants (Beker et al., 1989; for a review see Raguso, 2001). Consequently, natural selection in some species may act on buds and corollas in a similar fashion, resulting in similar volatile emissions.

We detected variation in relative floral scent production not only between *I. aggregata* and between *P. foliosissimum* floral structures (i.e., between buds and corollas) but also among individual *I. aggregata* and individual *P. foliosissimum* (see Tables 1 and 2, variation around mean compound emission). Variation in relative compound production among samples may be due to abiotic conditions, such as small differences in temperature and relative humidity at the time of sample collection (Robacker et al., 1983, 1988). Furthermore, biotic conditions, such as plant genotype (Chang et al., 1988), flower age (Robacker et al., 1988), selection by mutualistic pollinators (Galen and Kevan, 1980; Galen and Newport, 1988), and/or induction by antagonistic herbivores (Turlings et al., 1998), could cause variation in relative scent production. We are currently investigating the degree to which abiotic and biotic conditions influence scent emission among individual *I. aggregata* and *P. foliosissimum*.

The bud samples for both *I. aggregata* and *P. foliosissimum* showed large pinene peaks. High concentrations of monoterpenes, such as α- and β-pinene, attract herbivorous and seed-eating insects in some systems (e.g., Chang et al., 1988; Jallow et al., 1999), but repel them in others (Harborne, 1993). The predispersal seed-predator fly, *Hylemya* sp., uses *I. aggregata* and *P. foliosissimum* as its sole hosts, ovipositing eggs in the developing buds of both species. *Hylemya* may use high concentrations of α- and/or β-pinene, perhaps in association with the combined bouquet of other compounds, as an identifier to find oviposition sites. Preliminary studies indicate that variation in pinene levels in *P. foliosissimum* affects *Hylemya* oviposition (B. Dorsett and N. Brown, unpublished data), and *Hylemya* oviposit on other nonhost Polemoniaceae with similar pinene signatures as *I. aggregata* and *P. foliosissimum* buds (B. Dorsett and R. E. Irwin, unpublished data). We found that *I. aggregata* buds produced relatively more α-pinene than β-pinene, while *P. foliosissimum* buds produced more β-pinene than α-pinene. It is not known how variation in the ratio of α- to β-pinene affects *Hylemya* attraction and larval success. Variation in the ratio of α- to β-pinene between *I. aggregata* and *P. foliosissimum* could play a role in reported *Hylemya* preference for *P. foliosissimum* over *I. aggregata* (Zimmerman et al., 1984); however, further manipulative studies of pinene are needed to test its importance in *Hylemya* attraction.

Despite similar volatile emissions by the buds of *I. aggregata* and *P. foliosissimum*, the volatiles emitted by the corollas of the two species were distinct. The corollas of *I. aggregata* were characterized by esters, terpenoids, and

ketones creating an apple-sweet odor, while the corollas of *P. foliosissimum* were characterized by terpenoids, alcohols, and aldehydes creating a sweet-clover odor. It is not surprising that *I. aggregata* and *P. foliosissimum* emit different odors from their petal tissue, as the two species have different primary pollinators. *Ipomopsis aggregata* is pollinated primarily by hummingbirds, while *P. foliosissimum* is pollinated primarily by bumblebees. Although there is little evidence to suggest that hummingbirds use odor to locate flowers (for a review see Faegri and van der Pijl, 1979; Proctor et al., 1996), there is increasing experimental work suggesting that corolla odors play a role in the attraction of insect pollinators (Williams, 1983; Galen and Newport, 1988; Pellmyr, 1986; Dobson et al., 1999; Pelz, 1997; for a review see Raguso, 2001) as well as other insect visitors (i.e., Galen, 1983; Baldwin et al., 1997). For example, foraging bee pollinators use floral odors at close distance for orientation and floral selection, landing, and feeding (e.g., von Frisch, 1923; Lunau, 1992), and bumblebees bring odors from rewarding flower species back to the nest to convey to other nest members information about foraging success (Dornhaus and Chittka, 1999). Increased pollinator attraction based on fragrance chemistry drives the potential for positive directional selection on fragrance variation (Galen and Newport, 1988; Pellmyr, 1986; Pelz, 1997). It is possible that bumblebee pollinators could act as a strong selective agent on petal-tissue odors in *P. foliosissimum*.

The reason that *I. aggregata* maintains sweet-smelling flowers, even in the absence of selection by hummingbird pollinators, remains a mystery. It may be that the volatiles are merely by-products of essential biochemical pathways or that the volatiles are nonadaptive and occur in *I. aggregata* populations due to drift or phenotypic plasticity. Alternatively, it may be that the sweet odor is selected for in rare years where *I. aggregata* experiences pollination by bumblebees (primarily *B. appositus*) (Pleasants and Waser, 1985). In years where hummingbird densities are low in *I. aggregata* populations, nectar accumulates high in the tubular corollas of *I. aggregata* flowers, allowing *B. appositus* queens to skim nectar from flowers that reach a certain threshold. In the last two decades at the RMBL, there have been two seasons (1981 and 1997) where *B. appositus* queens were the primary pollinators of *I. aggregata* and were shown to be highly effective pollinators (Mayfield, Waser, and Price, unpublished data). Because bumblebees may rely on floral scents to locate and land on flowers, as described above, these rare bumblebee years could maintain sweet-smelling flowers in *I. aggregata* populations.

In summary, the corollas of *I. aggregata* are characterized by a more complex mixture of floral volatiles than the buds, although both are dominated by α-pinene. Additionally, buds of *P. foliosissimum* produce a volatile bouquet similar to that of *I. aggregata* buds, although the buds of the two species emit different ratios of α- to β-pinene. However, the corollas of *P. foliosissimum* emit different compounds from those of *I. aggregata*. The partition of volatiles between *I. aggregata* and *P. foliosissimum* buds and corollas and differences in volatile production between

I. aggregata and *P. foliosissimum* are consistent with selection pressures exerted by organisms interacting with these plants. More comparative studies on plants in the same family will help to elucidate whether similarities and differences in odor production are related to interacting organisms, phylogenetic relationships, and/or similar biochemical pathways. Our next step in this system is to experimentally manipulate the emission of volatile odors by the buds and corollas of *I. aggregata* and *P. foliosissimum* to examine the microevolutionary and macroevolutionary importance of odor emission to mutualistic and antagonistic visitors.

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LEARNING OF HERBIVORE-INDUCED AND NONSPECIFIC PLANT VOLATILES BY A PARASITOID, *Cotesia kariyai*

JUNJI FUKUSHIMA,[1] YOOICHI KAINOH,[1,*] HIROSHI HONDA,[1] and JUNJI TAKABAYASHI[2,3]

[1]*Institute of Agriculture and Forestry*
University of Tsukuba Tsukuba, Ibaraki 305-8572, Japan
[2]*Center for Ecological Research*
Kyoto University Otsuka 509-3, Hirano, Kamitanakami, Otsu 520-2113, Japan
[3]*CREST, JST (Japan Science and Technology Corporation)*

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Abstract—Learning of host-induced plant volatiles by *Cotesia kariyai* females was examined with synthetic chemicals in a wind tunnel. Wasps were preconditioned by exposure to volatiles and feces simultaneously. A blend of four chemicals, geranyl acetate, β-caryophyllene, (E)-β-farnesene, and indole, which are known to be specifically released from plants infested by host larvae *Mythimna separata* (host-induced blend), elicited a response in naive *C. kariyai*, but did not enhance the response after conditioning. A blend of five chemicals, (E)-2-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexen-1-yl acetate, β-myrcene, and linalool, which are known to be released not only from plants infested by the host larvae, but also from artificially damaged plants or undamaged ones (unspecific blend), elicited little response in naive wasps, but significantly enhanced the wasps' response after conditioning. With a blend of the above nine chemicals, wasps could learn the blend at lower concentrations than they did in the nonspecific blend. Hence, both the host-induced and nonspecific volatile compounds appear to be important for *C. kariyai* females to learn the chemical cues in host location.

Key Words—*Cotesia kariyai*, associative learning, wind tunnel, conditioning, host-induced plant volatiles, blend, synthetic chemicals, host location.

INTRODUCTION

Cotesia kariyai Watanabe (Hymenoptera: Braconidae) is a dominant parasitoid species of the common armyworm *Mythimna separata* (Walker) (Lepidoptera: Noctuidae). The wasp is a gregarious, endoparasitoid of second to early sixth

*To whom correspondence should be addressed. E-mail: parasite@sakura.cc.tsukuba.ac.jp

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instars of *M. separata* (Sato and Tanaka, 1984) and suppresses their population density in agricultural fields (Hirai, 1984). *M. separata* is widely distributed in Australia, New Zealand, and Asian tropic and temperate zones. In these areas, *M. separata* is an important pest of various Graminae crops (Kanda, 1991).

C. kariyai females use chemical cues of host and host-plant complex origin to find their hosts (Takabayashi and Takahashi, 1986; Takabayashi et al., 1991, 1995). *C. kariyai* has an innate preference for host-induced plant volatiles in Y-tube olfactometer bioassays: they prefer volatiles from plants infested with young host larvae to those from uninfested plants (Takabayashi et al., 1995). *C. kariyai* shows intensive antennation behavior in response to host by-products, such as feces, feeding traces, exuviae, and substances on the larval body surface that contain 2,5-dialkyltetrahydrofurans as kairomones (Takabayashi and Takahashi, 1986; Ohara et al., 1996). However, naive *C. kariyai* shows no response to the odor flow of host feces and the host itself in an Y-tube olfactometer (Takabayashi et al., 1995).

C. kariyai females have an ability to learn to associate host-induced plant volatiles with host feces (Fukushima et al., 2001). Wasps increased their flight response to host-induced plant volatiles in a wind-tunnel bioassay after conditioning, during which wasps were exposed simultaneously to host feces and volatiles. Many studies have revealed that parasitoids can learn to associate with their hosts various stimuli derived from plants (Turlings et al., 1993; Godfray, 1994; Vet et al., 1995).

Despite many papers on behavioral responses related to learning in parasitoids, there are few that focus on the plant components that parasitoids can learn (Turlings et al., 1991; Du et al., 1998; Vet et al., 1998). We have identified volatiles emitted from host-infested corn plants (Takabayashi et al., 1995). In this study, we classified the induced chemicals into two groups: (1) host-induced volatiles or volatiles that were specifically released from plants infested by host larvae *M. separata*, and (2) nonspecific volatiles or volatiles that were released not only from plants infested by the host larvae, but also from artificially damaged or undamaged plants. By using synthetic chemicals, we investigated which group of volatiles could be learned by *C. kariyai* under laboratory conditions.

METHODS AND MATERIALS

Insects. A colony of *C. kariyai* and its host, *M. separata*, were obtained from a stock culture at the University of Tsukuba. Both species were maintained at $25 \pm 1^{\circ}$C, $60 \pm 20\%$ relative humidity with a 16L: 8D photoperiod (on: 05:00 hr, off: 21:00 hr). Host larvae were reared on artificial diet in a similar manner as described by Kanda (1991). To maintain the wasp colony, third- to fifth-instar *M. separata* larvae were exposed to female wasps for oviposition. After wasp egression, cocoon masses were individually kept in glass test tubes (2 cm diam. $\times$ 20 cm

long) and emerging adult wasps were allowed to mate, which generally occurs right after emergence of the wasps. One day after emergence, females were transferred to a plastic cage (15 × 17 × 24 cm) with wet cotton on the bottom and honey on the wall. One- to 5-day-old females were used in all experiments.

Chemicals. We used nine commercially available chemicals to prepare the synthetic blends, including geranyl acetate, β-myrcene (Nakarai Chemicals, LTD., Kyoto, Japan), β-caryophyllene, (E)-β-farnesene, indole, (E)-2-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexen-1-yl acetate, and linalool (Wako Pure Chemical Industries, Ltd., Osaka, Japan). These compounds were released in relatively higher dosages from host-induced plants, and/or artificially damaged ones than nondamaged ones (Takabayashi et al., 1995).

Three types of blends were prepared by dissolving different kinds of synthetic chemicals in (1 ml) hexane. The amounts of each compound were determined based on their relative percentage amounts in the headspace of the corn plants infested by first/second-instar *M. separata* (Takabayashi et al., 1995). We called a blend of four chemicals that are specifically released from the plants infested by first/second-stadium host larvae [i.e., geranyl acetate, β-caryophyllene, (E)-β-farnesene, and indole] the host-induced blend (Figure 1A). We called a blend of five chemicals that are released not only from plants infested by first/second-stadium host larvae but also from artificially damaged or undamaged plants [i.e., (E)-2-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexen-1-yl acetate, β-myrcene, linalool] the nonspecific blend (Figure 1B). Furthermore, we called a blend of the above nine chemicals a mixed blend. Each blend was diluted to concentrations of 10^{-1} to 10^{-5} times with hexane, and these dilutions were used for conditioning and odor source.

Conditioning. Conditioning of *C. kariyai* to a blend together with their antennal contact to host feces was performed in a Petri dish (9 cm diam.). Each blend (10 μl), which was the same blend as used for the odor source of the wind tunnel bioassays, was applied to filter paper (1 × 1 cm) and placed in a Petri dish after evaporation of the solvent. Two sheets of nylon gauze were placed over the filter paper, thus preventing the wasps from contacting the filter paper directly. A fecal pellet excreted within 24 hr by fifth/sixth-stadium host larvae was placed on the nylon gauze. Female wasps were released into the Petri dish and the dish capped. When the wasps encountered the feces, they showed a characteristic antennal contact response. A female was allowed to contact the host feces for 1 min. After conditioning, the female was carefully collected with an insect aspirator and placed in another rearing cage (15 × 17 × 24 cm) until the wind-tunnel bioassay.

Bioassay. A wind tunnel (50 × 50 × 150 cm) was used to observe flight responses of wasps within 2 hr of conditioning. Flight responses were tested at a wind speed of 20–30 cm/sec at 25 ± 1°C, 60 ± 20% relative humidity and ca. 2200 lux. As an odor source, a filter paper (2 × 4 × 4 cm triangular) treated with a blend (20 μl) was placed on a stage (15 × 15 cm, 16 cm high) in a wind tunnel. Each conditioned female was introduced into a Petri dish (4 cm diam.) and

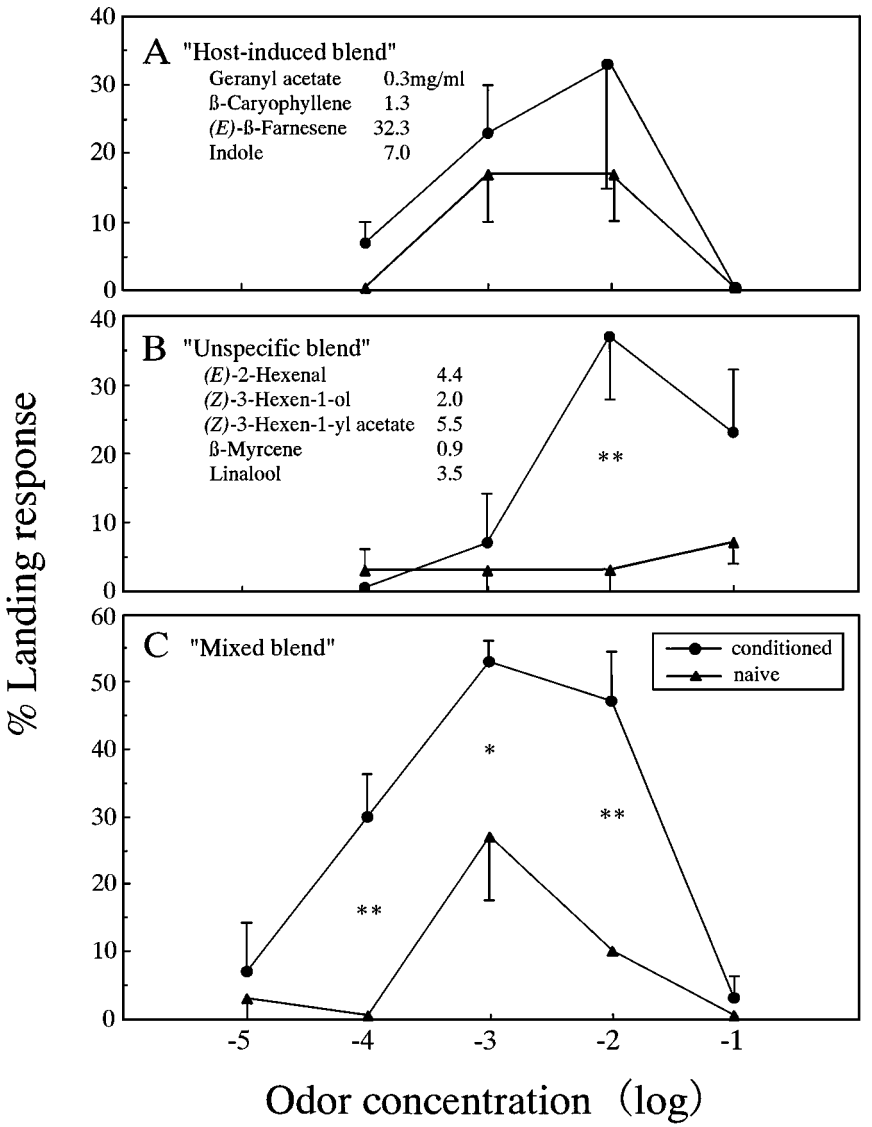


FIG. 1. Effect of a previous experience on subsequent flight response to (A) host-induced blend, (B) nonspecific blend, and (C) mixed blend by *Cotesia kariyai* in a wind tunnel. Dose–response was investigated at 10^{-1} to 10^{-5} concentrations. Error bars represent SE of three replicates (10 females $\times$ 3). Asterisks indicate significant difference by chi-square test ($^{*}P < 0.05$; $^{**}P < 0.01$).

released from a stage (8 × 8 cm, 20 cm high) at 15 cm downwind from the odor source. This distance was based on the previous work of Fukushima et al. (2001). Response of naive and conditioned females to different concentrations of the same blend was tested on the same day. The behavior of females was observed until they landed on the source or on the stage less than 5 cm away from the source within 5 min. When a female did not show any flight response during the test, she was considered to be not attracted by the odor source. A total of 30 females for each treatment were tested on three different days (10 females × 3).

RESULTS AND DISCUSSION

While the naive female *C. kariyai* responded to the host-induced blend (0–17%) (Figure 1A), they responded less to the nonspecific blend (3–7%) (Figure 1B). Responses were stronger to a mixed blend (27%) than to the other two blends at 10^{-3} concentration (Figure 1C). The response of naive wasps to the host-induced blend and to the mixed blend was not significantly different at each concentration ($P > 0.05$). These data suggest that there is little or no synergistic effect of the nonspecific blend to the host-induced blend on naive *C. kariyai* innate response.

The response of conditioned *C. kariyai* to the host-induced blend was not significantly higher than that of naive females at all concentrations ($P > 0.05$) (Figure 1A). By contrast, the response of conditioned females to the unspecific blend (37%) was higher than naive females (3%), but only at the 10^{-2} concentration of the blend ($P < 0.05$) (Figure 1B). Finally, the landing response of conditioned females to the mixed blend (30, 53, and 47%) was higher than that of naive females (0, 27, and 10%) between the 10^{-4} and 10^{-2} concentrations of the blend ($P < 0.05$) (Figure 1C). These data indicate that the wasp could learn the nonspecific blend and the mixed blend and that conditioned wasps responded to a lower concentration with the mixed blend than with the nonspecific blend. Thus, the host-induced blend would have a synergistic effect on learning response. Examples of synergistic effects of plant components have been previously reviewed in oviposition and feeding behavior in lepidopterous insects (Bernays and Chapman, 1994).

The maximum observed landing rate in *C. kariyai* is about 50% in our wind-tunnel experiment (Figure 1C). Naive insects showed a relatively high response (17%) to the host-induced blend when compared to the unspecific blend (Figure 1A); learning would, therefore, have less effect on the response to this blend, or the increase in responsiveness might not be detected under our experimental conditions. In contrast, the nonspecific blend elicited a low response in the naive wasps (Figure 1B), and the learning would therefore have more of an effect on the response to this blend.

C. kariyai was shown to learn blends of several synthetic chemicals from whole plant volatile components (Figure 1B,C). Similarly, other parasitoids can

learn some synthetic chemicals from whole plant volatiles [e.g., *C. marginiventris*, (Turlings et al., 1991); *Aphidius ervi*, (Du et al., 1998); *Leptopilina heterotoma*, (Vet et al., 1998)]. For example, *C. marginiventris* can learn a blend of 11 chemicals, i.e., (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-yl acetate, linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, indole, α-*trans*-bergamotene, (*E*)-β-farnesene, (*E*)-nerolidol, and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, when the wasps experienced the blend with host feces (Turlings et al., 1991). In *A. ervi*, previous experience with the plant–host complex increased the flight responses of females to single synthetic chemicals such as linalool, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-yl acetate, (*E*)-β-ocimene, and 6-methyl-5-hepten-2-one that are part of the volatile blend emitted by host-infested plants (Du et al., 1998). *L. heterotoma* also learns single plant component such as (*Z*)-3-hexen-1-ol associated with oviposition into the host (Vet et al., 1998).

Different mixtures of plant volatile components will influence the wasps' responses. In *C. marginiventris*, when host-induced plant volatiles were divided into four fractions according to volatility and the responses of the naive wasps to different blends of the four fractions were compared, fractions that contained indole and several sesquiterpenes were highly attractive to wasps in a wind tunnel (Turlings and Fritzsche, 1999). We divided volatiles from host-infested plants into a host-induced blend and a nonspecific blend and found that the responses of *C. kariyai* females were different for the two blends. Several major host-induced volatile compounds that were not commercially available were not tested. However, since the mixed blend used in this study elicited responses from both naive and conditioned wasps equivalent to their response to the infested leaf extract (Fukushima et al., 2001), our results using synthetic chemicals may partly explain the natural phenomena. Further study using complete blends of host-induced plant volatiles need to be done.

It is well-accepted that plants infested by herbivores attract carnivorous natural enemies of herbivores (Takabayashi and Dicke, 1996; Dicke and Vet, 1999). Special attention has been paid in plant–carnivore interactions to volatile compounds that are specifically produced by plants in response to herbivory (herbivore-induced plant volatiles) (Dicke, 1999; Turlings and Fritzsche, 1999). However, in the natural habitat, when *C. kariyai* finds host feces on a plant, the insect is surrounded not only by host-induced plant volatiles, but also by nonspecific ones. If the host density is low and the concentration of host-induced volatiles is accordingly low, associative learning of nonspecific volatiles with antennal contact with host feces would favor the wasp in the next host–microhabitat finding, rather than ignoring a potential habitat. A similar situation was discussed by Powell et al. (1998), in which *A. ervi* responded to an undamaged plant in a wind tunnel after experiencing the plant/host complex.

In our study, the role of each component was not determined. It would be necessary to test the learning response to each individual compound and also

to the blend with the individual compounds removed. These data would further elucidate the degree of specificity of the learned response and may help to reveal the role of volatile chemicals in parasitoid learning.

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IDENTIFICATION OF A TRITERPENOID SAPONIN FROM A CRUCIFER, *Barbarea vulgaris*, AS A FEEDING DETERRENT TO THE DIAMONDBACK MOTH, *Plutella xylostella*

TETSURO SHINODA,[1,*] TSUNEATSU NAGAO,[2] MASAYOSHI NAKAYAMA,[3] HIROAKI SERIZAWA,[4] MASAJI KOSHIOKA,[3] HIKARU OKABE[2], and AKIRA KAWAI[1]

[1]*National Institute of Vegetable and Tea Science 360, Ano, Mie, 514-2392, Japan*

[2]*Faculty of Pharmaceutical Science, Fukuoka University 8-19-1, Nanakuma, Jonan-ku, Fukuoka, 814-0180, Japan*

[3]*National Institute of Floricultural Science 2-1, Fujimoto, Tsukuba, Ibaraki, 305-8519, Japan*

[4]*Nagano Vegetable and Ornamental Crops Experiment Station 2206, Oomuro, Matsushiro, Nagano, Nagano, 381-1211, Japan*

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Abstract—Larvae of the diamondback moth, *Plutella xylostella*, a crucifer specialist, refuse to feed on a crucifer, *Barbarea vulgaris*, because of the presence of a feeding deterrent, which is extractable with chloroform. We isolated a feeding deterrent from *B. vulgaris* leaves, by successive fractionations with silica-gel, ODS, i.e., C_{18} reversed phase, and Sephadex LH-20 column chromatographies, and ODS-HPLC, guided by a bioassay for feeding deterrent activity. The structure of the compound was determined to be a monodesmosidic triterpenoid saponin, 3-*O*-[*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]-hederagenin, based on FAB-MS, ^{1}H- and ^{13}C-NMR spectra, and hydrolysis experiments. When the compound was applied to cabbage leaf disks at greater than 0.18 μg/mm^2, consumption of the disks by third instars was less than 11% of control disks treated with the solvent alone. Furthermore, all first instars died on the disks treated with the same concentrations. Because the concentration of the compound in the fresh leaves of *B. vulgaris* was comparable to the effective dose in the cabbage leaf disk tested, we conclude that the unacceptability of *B. vulgaris* to *P. xylostella* larvae is primarily due to this saponin.

* To whom correspondence should be addressed. E-mail: shinoda@naro.affrc.go.jp

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Key Words—*Barbarea vulgaris*, Brassicaceae, diamondback moth, feeding deterrent, 3-*O*-[*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]-hederagenin, host plant resistance, insect-plant interactions, Lepidoptera, *Plutella xylostella*, triterpenoids, saponins.

INTRODUCTION

The diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae), a worldwide pest on cruciferous crops, is a typical crucifer specialist. The intimate relationship between crucifers and *P. xylostella* is mainly mediated by glucosinolates and their breakdown products, isothiocyanates, which stimulate oviposition of the adults and feeding of the larvae (Thorsteinson, 1953; Gupta and Thorsteinson, 1960a,b; Hillyer and Thorsteinson, 1969). However, most, but not all, crucifers containing glucosinolates are suitable hosts for *P. xylostella*; the wintercress, *Barbarea vulgaris* R. Br. (Brassicaceae), is a rare exception.

B. vulgaris is a biennial or perennial herbaceous plant of European origin and has recently been naturalized in Japan. The leaves of *B. vulgaris* contain (2*R*)- and (2*S*)-glucobarbarin and glucobrassicin, as major glucosinolates that stimulate the oviposition of *Pieris rapae*, another crucifer specialist (Huang et al., 1994). *P. xylostella* adults also preferably lay eggs on this plant, probably being stimulated by the glucosinolates. Despite the presence of glucosinolates, hatched larvae refuse to feed on the plant and cannot develop to the second instar (Idris and Grafius, 1996). In a previous study, we found strong feeding deterrent activity to *P. xylostella* larvae in a chloroform-extract of *B. vulgaris* leaves (Serizawa et al., 2001).

We now report the isolation and identification of a triterpenoid saponin from the leaves of *B. vulgaris* that strongly deters feeding of *P. xylostella* larvae. To our knowledge, this is not only the first feeding deterrent to *P. xylostella* found in the family Brassicaceae, but also the first oleanane-type saponin found in this family.

METHODS AND MATERIALS

Insects and Plants. Larvae of the diamondback moth, *Plutella xylostella*, a strain susceptible to insecticides, were maintained on radish seedlings at 25°C and 12L:12D following the method of Yamada and Koshihara (1978). *Barbarea vulgaris* var. *variegata* (Chiltern Seeds) and the cabbage, *Brassica oleracea* L. cv.YRSE, were grown in a greenhouse without any application of insecticides.

Isolation of Feeding Deterrent. Isolation of the feeding deterrent from *B. vulgaris* leaves was performed by a series of column chromatographies and ODS-HPLC, guided by successive bioassays of the fractions (Figure 1). Fresh leaves of *B. vulgaris* (2.8 kg) were homogenized in MeOH (14 liters), extracted overnight at room temperature, and filtered. After evaporation of MeOH, the aqueous residue was extracted with $CHCl_3$. The $CHCl_3$ extract (38.1 g) was chromatographed on silica gel (Kieselgel 60, Merck; 500 × 55 mm ID), eluted

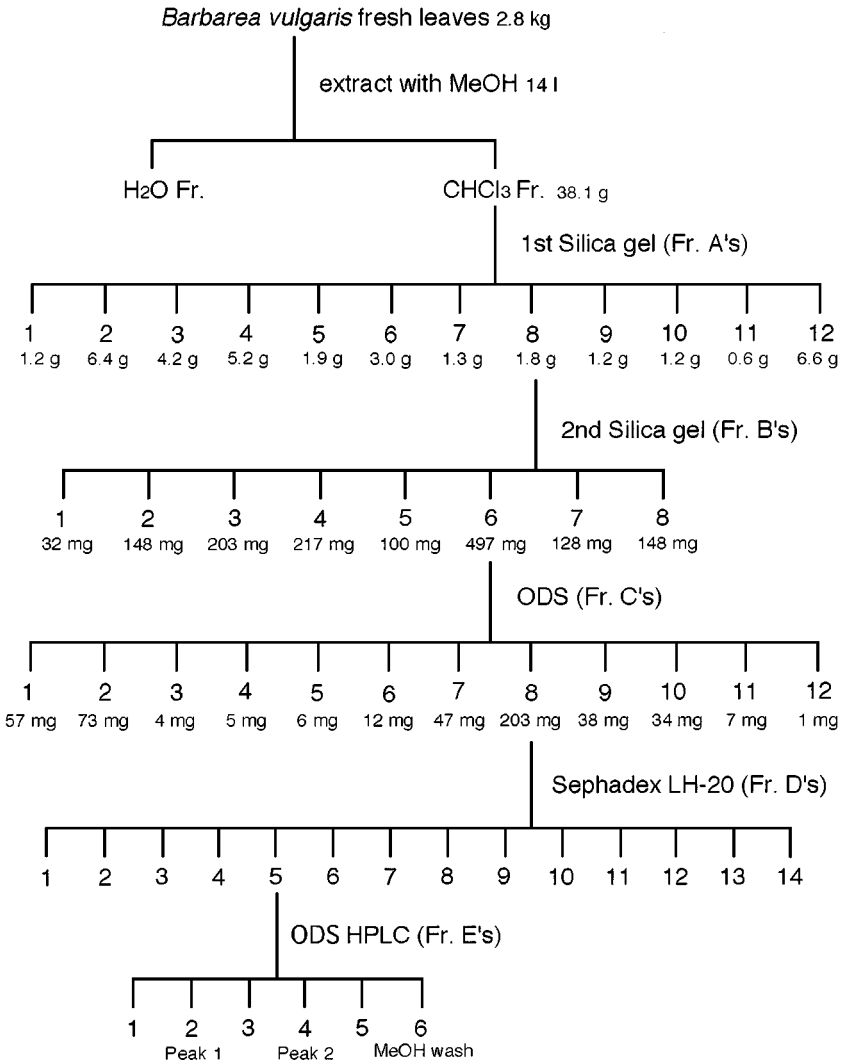


FIG. 1. Schematic diagram of the procedure for isolating the feeding deterrent from *B. vulgaris*.

successively with 0, 5, 10, 15, 20, and 100% MeOH in $CHCl_3$, and the eluate was collected (initially 100 ml/subfraction). Each subfraction was analyzed by TLC (plate, 5554, Merck; solvent system, $CHCl_3$–MeOH–H_2O, 32:9:1; development, spraying with 10% H_2SO_4 followed by heating), and subfractions showing similar spotting patterns were combined, providing 12 fractions (Fr. A-1 to A-12).

The solvents corresponding to the fractions are as follows: A-1, 100% $CHCl_3$, 1500 ml; A-2, 100% $CHCl_3$, 3500 ml; A-3, 5% MeOH/$CHCl_3$, 1000 ml; A-4, 5% MeOH/$CHCl_3$, 4000 ml; A-5, 10% MeOH/$CHCl_3$, 1000 ml; A-6, 10% MeOH/$CHCl_3$, 2000 ml; A-7, 10% MeOH/$CHCl_3$, 2300 ml; A-8, 10% MeOH/$CHCl_3$, 700 ml and 15% MeOH/$CHCl_3$, 2000 ml; A-9, 15% MeOH/$CHCl_3$, 1500 ml; A-10, 15% MeOH/$CHCl_3$, 2500 ml; A-11, 20% MeOH/$CHCl_3$, 2000 ml; A-12, 100% MeOH, 2500 ml. The most active fraction, A-8, was applied again to a silica gel column (Kieselgel 60, Merck; 200 $\times$ 25 mm ID) and successively eluted with 0, 5, 10, 12.5, 15, 20, and 100% MeOH in $CHCl_3$. The eluate was collected (50 ml/subfraction), analyzed by TLC, and combined into eight fractions (Fr. B-1 to B-8) in a manner similar to the first separation. The solvents corresponding to the B fractions are as follows: B-1, 100% $CHCl_3$, 400 ml, 5% MeOH/$CHCl_3$, 400 ml, and 10% MeOH/$CHCl_3$, 100 ml; B-2, 10% MeOH/$CHCl_3$, 300 ml; B-3, 12.5% MeOH/$CHCl_3$, 150 ml; B-4, 12.5% MeOH/$CHCl_3$, 200 ml; B-5, 12.5% MeOH/$CHCl_3$, 50 ml and 15% MeOH/$CHCl_3$, 50 ml; B-6, 15% MeOH/$CHCl_3$, 350 ml; B-7, 15% MeOH/$CHCl_3$, 500 ml; B-8, 20% MeOH/$CHCl_3$, 400 ml and 100% MeOH 400 ml. The active fraction, B-6, was chromatographed on ODS (Cosmosil, Nacalai; 100 $\times$ 30 mm ID), successively eluted with 150 ml of 30, 40, 45, 50, 55, 60, 65, 70, 80, 90, and 100% MeOH in H_2O, and 100% $CHCl_3$, providing 12 fractions (Fr. C's). The active fraction, C-8, was applied to Sephadex LH-20 (Pharmacia, 1000 $\times$ 25 mm ID) and eluted with 80% MeOH, providing 14 fractions (50 ml/fraction) (Fr. D's). The active fraction, D-5, was fractionated by ODS-HPLC (column, SenshuPak ODS-4253-D, 250 $\times$ 10 mm ID; column temperature, 40°C; solvent, 80% MeOH, 3 ml/min; detection, UV 210 nm). Fr. D-5 showed a major peak 1 (retention time, 10.1 min), that was designated as compound **1,** and a minor peak 2 (retention time, 14.9 min). The peak area of peak 2 was about 1/20 that of peak 1. Six fractions (Fr. E's) corresponding to prepeak 1, peak 1, postpeak 1, peak 2, postpeak 2, and the MeOH wash of the column were collected separately for the bioassay.

*Chemical Identification of Compound **1***. Compound **1** was obtained from Fr. D-5 by preparative HPLC (column, Cosmosil 5C18AR, 250 $\times$ 20 mm ID, Nacalai; solvent, 82% MeOH; flow rate, 7 ml/min; detection, RI). The yield of compound **1** was 124 mg out of 151 mg of Fr. D-5.

FAB-MS, ^{1}H-NMR, ^{13}C-NMR, and Optical Rotation. For structural analysis of compound **1**, a fast atom bombardment mass spectrum (FAB-MS) was obtained using a Jeol HX-110 mass spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Jeol A-500 spectrometer. Optical rotation was measured on a JASCO DIP-360 polarimeter.

Methanolysis and GLC Analysis of Methyl Glycoside. Compound **1** (2 mg) was dissolved in 1 N HCl/MeOH (1 ml) and heated to 95°C for 2 hr. After cooling, the acidic solution was neutralized with Ag_2CO_3. $AgCl_2$ and Ag_2CO_3 were centrifuged off, and the supernatant was analyzed by TLC with authentic hederagenin

(Extrasynthese) (solvent system, $CHCl_3$–MeOH, 19:1) and methyl glycoside (solvent system, $CHCl_3$–MeOH–H_2O, 15:6:1). The supernatant described above was dried under a N_2 current, and the residue was dissolved in trimethylsilyl imidazole (0.2 ml) to convert the methyl glycosides into TMS ethers. After incubation at 95°C for 10 min, the solution (1 μl) was analyzed by GLC (Shimadzu GC-14A with FID) to identify the sugar. Sugar standards were treated in the same manner. GLC conditions were as follows: column, DB-1, 0.25 mm × 30 m, J & B Scientific; carrier gas, helium; linear velocity in column, 300 mm/sec; split ratio, 1/40; column temperature, 170°C; injection port temperature, 250°C; FID detector temperature, 250°C.

Bioassays with Third-Instar Larvae. Fractions from *B. vulgaris* and the purified compound **1** were dried in vacuo, dissolved in 80% MeOH, and applied topically to both sides (25 μl on each side) of cabbage leaf disks (19 mm diam., ~100 mg). For controls, solvent (80% MeOH) alone was applied to leaf disks. After evaporation of the solvent, the disks were placed individually at the center of moist filter paper set in a plastic container (60 mm diam. × 30 mm high) and three third instars of *P. xylostella* were gently transferred onto the leaf disks. The containers were closed with a lid and kept at 25°C and a 12L:12D cycle. After 24 hr of incubation, the disks were scanned with a digital image scanner (ES-2000, Epson) and the area consumed was measured by using imaging analysis software (NIH image). Assays were performed with five replicates.

Bioassays with First-Instar Larvae. Cabbage leaf disks were treated with various concentrations of compound **1** dissolved in 80% MeOH or the solvent alone (control) as described above. Each disk was set on moist filter paper (25 mm diam.) in a glass dish (28 mm diam. × 15 mm high). Five first instars within 12 hr of hatching were transferred gently onto the leaf disk with a fine brush. The dishes were sealed with Parafilm and kept at 25°C and a 12L:12D cycle. After 24 hr of incubation, the survival of *P. xylostella* larvae was examined under a microscope. Larvae that did not move after being touched with a pinhead were counted as dead. Assays were performed with 10 replicates.

Statistical Analysis. The significance of the difference of the consumption or survival rate between the test and the control samples was analyzed by an independent *t*-test (two-way) using commercial software (Statistica, StatSoft).

*Quantification of Compound **1** in* B. vulgaris *Leaves.* Fresh matured leaves of *B. vulgaris* were weighed and extracted three times with 10 volumes of 80% MeOH (v/w). The 80% MeOH extract (3 ml) was loaded on a Sep-Pak Plus C18 cartridge (Waters) that was equilibrated with 80% MeOH, and eluted with 80% MeOH (10 ml × 3). The whole eluate was dried in vacuo, and the residue was dissolved in 1 ml of 80% MeOH–0.1% CH_3COOH solution, and 100 μl of the solution [10 mg fresh weight equivalent (FWE)] was analyzed by analytical HPLC (Intersil ODS-2, 250 × 4.6 mm ID; column temperature, 40°C; solvent, 80% MeOH–0.1% CH_3COOH; flow rate, 0.6 ml/min; detection, UV 210 nm). Compound **1** eluted at

a retention time of 10.1 min. Concentration was calculated based on the peak area of the sample compared with purified compound **1**.

RESULTS

Isolation of Feeding Deterrent. In a previous paper, we reported that the $CHCl_3$ fraction of the MeOH extract of *B. vulgaris* leaves showed strong feeding deterrent activity against larvae of *P. xylostella* (Serizawa et al., 2001). To isolate the feeding deterrent, we fractionated the $CHCl_3$ fraction by several chromatographic steps guided by no-choice bioassays (Figure 1). After the first silica gel chromatography, when applied at a high dose to cabbage leaf disks (1120 mg FWE/disk), Fr. A-2, A-7, and A-8 showed strong ($P < 0.001$), Fr. A-3 moderate ($P < 0.01$), and Fr. A-6 and A-9 weak ($P < 0.05$) feeding deterrent activity (Figure 2A). When the fractions were applied at a low dose (140 mg FWE/disk),

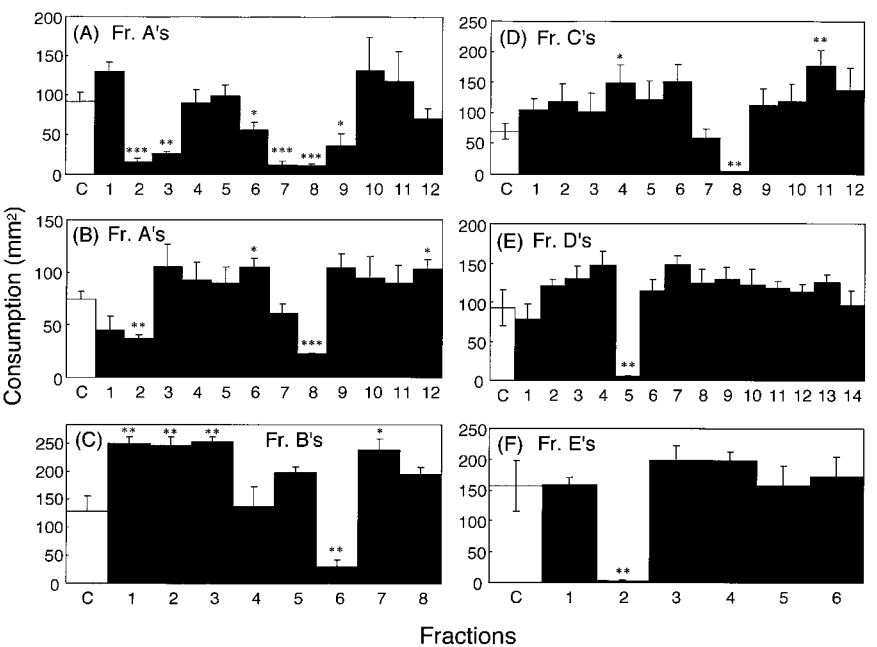


FIG. 2. Effects of *B. vulgaris* fractions on the feeding of *P. xylostella* larvae. The doses applied to cabbage leaf disks were 1120 (A), 140 (B), 700 (C), and 1400 mg FWE (D, E, F). Control disks were treated with 80% MeOH alone. Open and solid bars indicate consumption of control and test disks, respectively (means ± SE, $N = 5$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

only Fr. A-8 ($P < 0.001$) and Fr. A-2 ($P < 0.01$) showed significant feeding deterrent activity (Figure 2B). We further purified only the most active fraction, A-8, on a second silica gel column, finding feeding deterrent activity in Fr. B-6 ($P < 0.01$) at 700 mg FWE/disk (Figure 2C). When this fraction was separated on an ODS column, feeding deterrent activity was found only in Fr. C-8 at 1400 mg FWE/disk ($P < 0.01$) (Figure 2D). Separation of this fraction on a Sephadex LH-20 column resulted in isolation of only one active fraction, D-5 (1400 mg FWE/disk, $P < 0.01$) (Figure 2E). After purification of Fr. D-5 by ODS-HPLC, only Fr. E-2 showed feeding deterrent activity ($P < 0.01$) at 1400 mg FWE/disk (Figure 2F).

*Chemical Identification of Compound **1***. Compound **1** was purified from Fr. D-5 as a white powder by a preparative ODS-HPLC. Optical rotation, FAB-MS, ^{1}H-NMR and ^{13}C-NMR spectra of compound **1** were as follows. $[\alpha]_D^{25} + 38.7^\circ$ ($c = 1.4$, pyridine). Negative ion FAB-MS (glycerol matrix) *m/z*: 795.4535 ([M–H]$^-$; $C_{42}H_{67}O_{14}$ requires 795. 4531), 633 ([M–glc-H]$^-$), 471 ([M–2glc-H]$^-$). ^{1}H-NMR δ (C_5D_5N): 0.93 (6H, s, C-25-H and C-29-H), 0.96 (3H, s, C-24-H), 1.00 (3H, s, C-30-H), 1.02 (3H, s, C-26-H), 1.25 (3H, s, C-27-H), 3.28 (br. d, $J = 11$, C-18-H), 3.69 (br.d, $J = 11$, C-23-Ha), 3.85 [ddd, $J = 2, 4, 9$, C-5′-H], 3.96 [ddd, $J = 2, 6, 9$, C-5″-H], 4.00 (dd, $J = 8, 9$, C-2′-H), 4.04 (dd, $J = 8, 9$, C-2″-H), 4.14 (dd, $J = 9, 9$, C-4″-H), 4.18 (2H, dd, $J = 9, 9$, C-3′-H and C-3″-H), 4.20 (dd, $J = 4, 10$, C-3-H), 4.25 (dd, $J = 6, 12$, C-6″-Ha), 4.29 (dd, $J = 9, 9$, C-4′-H), 4.41 (dd, $J = 2, 12$, C-6′-Ha), 4.49 (dd, $J = 2, 12$, C-6″-Hb), 4.52 (dd, $J = 4, 12$, C-6′-Hb), 5.04 (d, $J = 8$, C-1′-H), 5.14 (d, $J = 8$, C-1″-H), 5.47 (br. s, C-12-H). ^{13}C-NMR δ (C_5D_5N): 13.6 (C-24), 16.1 (C-25), 17.5 (C-26), 18.2 (C-6), 23.6 (C-16), 23.7 (C-30), 23.8 (C-11), 25.7 (C-2), 26.1 (C-27), 28.3 (C-15), 30.9 (C-20), 32.9 (C-7), 33.2 (C-22), 33.2 (C-29), 34.2 (C-21), 36.9 (C-10), 38.6 (C-1), 39.8 (C-8), 41.9 (C-18), 42.2 (C-14), 43.3 (C-4), 46.4 (C-19), 46.6 (C-17), 47.7 (C-5), 48.1 (C-9), 62.1 (C-6′), 62.3 (C-6″), 64.7 (C-23), 71.4 (C-4″), 74.6 (C-2″), 75.2 (C-2′), 76.3 (C-5′), 76.7 (C-3′), 78.0 (C-3″), 78.3 (C-5″), 81.3 (C-4′), 82.4 (C-3), 104.9 (C-1″), 105.3 (C-1′), 122.5 (C-12), 144.8 (C-13), 180.1 (C-28).

The molecular formula of compound **1** was established to be $C_{42}H_{68}O_{14}$ by high-resolution FAB-MS. The ^{1}H-NMR spectrum of compound **1** showed signals of six tertiary methyl groups [δ 0.93 (6H), 0.96, 1.00, 1.02, and 1.25], one trisubstituted olefinic proton (δ 5.47), and two sugar anomeric protons (δ 5.04 and 5.14). The ^{13}C-NMR spectrum revealed the presence of six saturated quaternary carbons (δ 30.9, 36.9, 39.8, 42.2, 43.3, and 46.6), a pair of olefinic carbons (δ 122.5 and 144.8), one carboxylic acid carbon (δ 180.1), three hydroxymethyl carbons (δ 62.1, 62.3, and 64.7), and two sugar anomeric carbons (δ 104.9 and 105.3). These spectral data indicated that compound **1** is a glycoside of olean-12-ene type triterpene carboxylic acid, having a hydroxymethyl group on the aglycone and two hexosyl groups as sugar moieties.

The methanolysate of compound **1** showed two spots that gave the same R_f values as hederagenin (solvent system, $CHCl_3$–MeOH, 19:1; $R_f = 0.31$) and

methyl glucoside (solvent system, $CHCl_3$–MeOH–H_2O, 15:6:1; $R_f = 0.26$) on TLC. Methyl glucoside was also identified by GLC as a TMS ether. The ^{13}C-NMR spectrum of compound **1**, and the signals of the aglycone moiety were consistent with the data reported for a hederagenin 3-*O*-glycoside (Kohda et al., 1990), indicating compound **1** is a glucobiosyl hederagenin. The HMBC spectrum was examined to determine the sugar linkage of compound **1**. The HMBC correlations, observed between the signals of C-3-H (δ 4.20) and C-1′ (C-1 of inner glucosyl group, δ 105.3), C-1′-H (δ 5.04) and C-3 (δ 82.4), C-4′-H (δ 4.29) and C-1″ (C-1 of terminal glucosyl group, δ 104.9), C-1″-H (δ 5.14) and C-4′ (δ 81.3), indicated a cellobiosyl group is linked to C-3 of hederagenin. From these data, compound **1** was identified as 3-*O*-[*O*-β-D-glucopyranosyl-(1 $\rightarrow$ 4)-β-D-glucopyranosyl] hederagenin, which has been reported only in the mature fruits of *Fatsia japonica* (Figure 3) (Aoki et al., 1976).

*Effect of Compound **1** on Feeding of Third-Instar Larvae.* Figure 4 shows the effect of compound **1** on feeding of third instars. Compound **1** deterred feeding of the larvae dose-dependently. The consumption of the cabbage leaf disks treated with 25 μg or more of compound **1** was less than that of the control ($P < 0.001$). Remarkably, the mean consumption of the disks treated with 50 and 125 μg was only 11% and 5%, respectively, of that of the control.

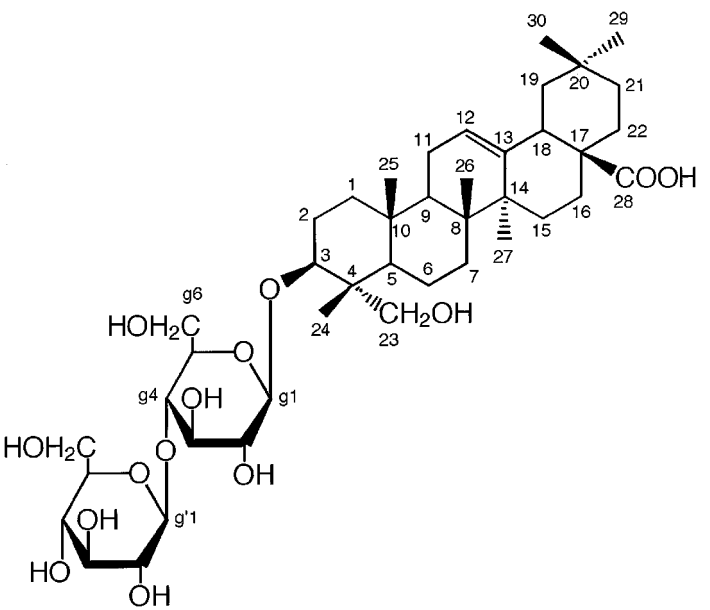


FIG. 3. Structure of the feeding deterrent (compound **1**) isolated from *B. vulgaris*.

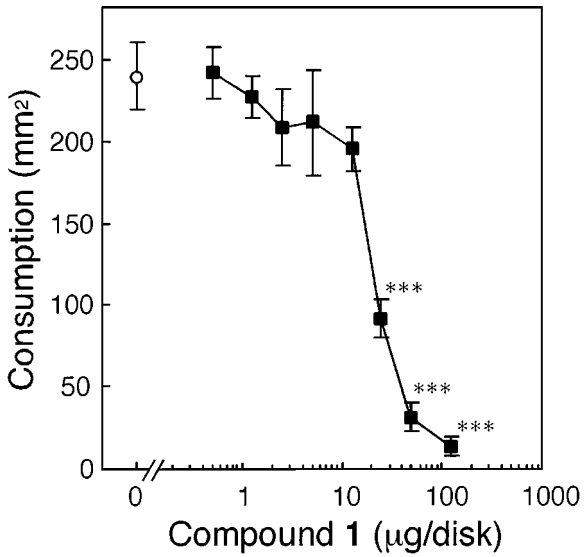


FIG. 4. Dose–response effect of compound **1** on the feeding of third-instar larvae. Data represent the consumption of the test (■) and control (○) leaf disks (mean ± SE, $N = 5$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

*Effect of Compound **1** on Survival of First-Instar Larvae.* Figure 5 shows the effect of compound **1** on the survival of the first instars. Compound **1** reduced the survival rate dose-dependently. The survival rates on the disks treated with 5 μg or more of compound **1** were significantly lower than the control value. Only 6% of the larvae could survive on the disks treated with 12.5 μg of compound **1**, and all larvae died on the disks treated with more than 50 μg.

*Quantification of Compound **1** in* B. vulgaris *Leaves.* The concentration of compound **1** in the fresh mature leaves of *B. vulgaris* was estimated as 1.1 ± 0.3 mg/g (mean ± SD, $N = 4$).

DISCUSSION

We isolated and identified a triterpenoid saponin, 3-*O*-[*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]-hederagenin (**1**), as a feeding deterrent to the diamondback moth, *Plutella xylostella*, from the winter cress, *Barbarea vulgaris*. It shows strong deterrence to this insect. Although a number of feeding deterrents to *P. xylostella* have been reported from various plant species, none has been found in the family Brassicaceae. Furthermore, few saponins have been reported from the family Brassicaceae (Hostettmann and Marston, 1995). To our knowledge,

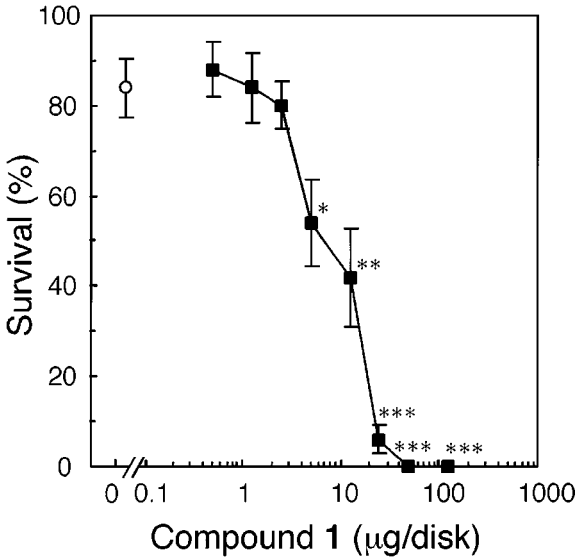


FIG. 5. Dose–response effect of compound **1** on the survival of first-instar larvae. Data represent the survival rate on the test (■) and control (○) leaf disks (mean ± SE, $N = 10$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

this is the first feeding deterrent to *P. xylostella* and the first oleanane-type saponin isolated from this family.

On leaf disks of *B. vulgaris*, the third instars of *P. xylostella* consumed less than 10% of those of cabbage, and none of the first instars developed to the second instar (Serizawa et al., 2001). When the cabbage leaf disks (average weight = ∼100 mg) were treated with 50 μg or more of compound **1**, similar effects were found on both third and first instars. Because the effective dose of compound **1** on cabbage leaf disks (>0.5 mg/g) is comparable to that found in the fresh leaves of *B. vulgaris* (1.1 mg/g), we conclude that compound **1** alone is sufficient to inhibit the feeding of *P. xylostella* larvae on *B. vulgaris*. Other feeding deterrents present in Fr. A-2, A-3 and A-7 may also contribute.

The ED_{50}, i.e., 50% effective dose of compound **1,** for the third instars was estimated to be 20 μg/disk (0.07 μg/mm^2). In no-choice feeding deterrent bioassays similar to our experiment, several quassinoids isolated from *Picrasma ailanthoides* (Simaroubaceae) showed strong activity at 50–200 μg/disk (0.16–0.64 μg/mm^2) against the third instars (Daido et al., 1993), and 14-deoxyandrographolide isolated from *Andrographis paniculata* (Acanthaceae) showed minimal activity at 100 μg/disk (0.32 μg/mm^2) against the fourth instars (Hermawan et al., 1997). The activity of compound **1**, thus, is as strong as that of feeding deterrents reported previously.

Glucosinolates present in crucifers generally function in defense against nonadapted insects. However, crucifer specialists counteradapt and even utilize glucosinolates as key stimulants for host selection. Other classes of secondary compounds in the restricted genera of crucifers have been found to deter feeding of some crucifer specialists: cucurbitacin E and cucurbitacin I in *Iberis amara* against the flea beetle, *Phyllotreta nemorum* (Nielsen et al., 1977), 2-*O*-β-D-glucopyranosyl cucurbitacin E in *Iberis amara* against the cabbage butterfly, *Pieris rapae* (Sachdev-Gupta et al., 1993a), and cardenolides in *Erysimum cheiranthoides* against *P. rapae* (Sachdev-Gupta et al., 1993b). Feeny (1977) referred to such substances as "a second line of chemical defense" in contrast with glucosinolates, "the first line of chemical defense". *B. vulgaris* contains glucosinolates (Huang et al., 1994) and is favorably selected for oviposition by *P. xylostella* in the field (Idris and Grafius, 1996). Therefore, compound **1** apparently functions as "the second line of chemical defense" against *P. xylostella* larvae.

Besides *P. xylostella*, some crucifer specialists, such as the pollen beetle, *Meligethes* sp. (Merker and Nilsson, 1995), and the flea beetle, *Phyllotreta nemorum* (Nielsen, 1996, 1997a,b, 1999), cannot infest *B. vulgaris*, suggesting the presence of unidentified feeding deterrents or toxins to these insects in this plant. It will be of interest to see whether compound **1** also deters their feeding or growth. *B. vulgaris* is, however, a good host for *P. rapae* (Huang et al., 1994), indicating that the feeding deterrent activity of compound **1** is species-specific to some extent.

Finally, we point out that compound **1** can be utilized to develop novel methods to control *P. xylostella* on cruciferous crops. Because of the outstanding ability of *P. xylostella* to develop resistance to a wide spectrum of insecticides, alternative control methods are now in great demand (Talekar and Shelton, 1993). Compound **1** or its derivatives might be used as repellents to protect susceptible cruciferous crops from infestation. Resistant cruciferous crops harboring compound **1** could be developed via various breeding techniques, e.g., traditional crossing, somatic hybridization, or genetic engineering. Because *B. vulgaris* belongs to the family Brassicaceae, it may be relatively easy to introduce pathways for the biosynthesis of compound **1** into cruciferous crops. Indeed, intertribal somatic hybrids between *B. vulgaris* and *Brassica napus* have already been generated (Fahleson et al., 1994). Furthermore, because *B. vulgaris* has been used as a vegetable and medicinal plant (Senatore et al., 2000), compound **1** is likely to be safe for humans.

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CHEMICAL DEFENSE IN THE PLANT BUG *Lopidea robiniae* (UHLER)

JOSEPH K. STAPLES,[1] BRYAN S. KRALL,[1] ROBERT J. BARTELT,[2] and DOUGLAS W. WHITMAN[1,*]

[1]*Behavior, Ecology, Evolution, and Systematics Section*
4120 Biological Sciences, Illinois State University
Normal, Illinois 61790

[2]*USDA, ARS National Center for Agricultural Utilization Research*
Bioactive Agents Research Unit
Peoria, Illinois 61604

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Abstract—Secretions from the metathoracic glands (MTG) of the black locust bug, *Lopidea robiniae* (Uhler) (Heteroptera: Miridae) contained six major compounds, including (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, (*E*)-2-octenal, (*E*)-2-octen-1-ol (*E*)-2-heptenal, and (*Z*)-3-octen-1-ol. Males and females did not differ significantly in the relative compositions of identified compounds. In feeding trials, six bird species [robin (*Turdus migratorious*), blue jay (*Cyanocitta cristata*), brown thrasher (*Toxostoma rufum*), killdeer (*Charadrius vociferus*), starling (*Sturnus vulgaris*), and house wren (*Troglodytes aedon*)] demonstrated feeding aversions towards *L. robiniae*, implying that black locust bugs are chemically defended. Bugs discharged the liquid contents of their MTG when attacked, thereby producing a strong and distinct odor. Some birds immediately ejected bugs out of their mouth after biting them, suggesting that the MTG secretion was a deterrent.

Key Words—Aldehydes, alcohols, chemical defense, Heteroptera, Miridae, *Lopidea robiniae*, *Robinia pseudoacacia*, (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, (*E*)-2-octenal, (*E*)-2-octen-1-ol, (*E*)-2-heptenal, (*Z*)-3-octen-1-ol.

INTRODUCTION

The mirids are the largest family of Heteroptera, numbering over 1900 species in North America (McPherson, 1989) and 10,000 species worldwide (Schuh and Slater, 1995). Mirids predominately use crypsis and agile escape maneuvers, such

* To whom correspondence should be addressed. E-mail: dwwwhitm@ilstu.edu

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as taking flight, dropping, and feigning death, to avoid predation (Aldrich, 1988). Although adults have metathoracic scent glands, (MTG) and the nymphs and some adults possess dorsal abdominal scent glands (DAG), the chemical ecology of only a few species has been examined (Aldrich, 1988; Millar et al., 1997; Millar and Rice, 1998; McBrien and Millar, 1999).

A common genus in the family Miridae is *Lopidea*, with more than 60 species occurring solely in the Western Hemisphere throughout much of North and Central America. *Lopidea* are different from other mirids in that most are relatively large (>5.0 mm) and are brightly colored with red, orange, or yellow on a black background, creating striking patterns (Asquith, 1993). For insects, large size and conspicuous coloration is often associated with chemical defense (Pasteels et al., 1983).

The black locust bug, *Lopidea robiniae* (Uhler), is common in the eastern United States and is found nearly everywhere its host tree, black locust, *Robinia pseudoacacia* L., grows. Adults are typically 6.0–6.5 mm in length and 2.1 mm in width. Males and females are aposematically colored orange–yellow with a broad black stripe that runs along the mid-dorsum from the tip of the head to the end of the abdomen (Knight, 1941). The nymphs are also aposematic with black wingpads on an orange–red body. Our observations suggest that, in Illinois, black locust bugs overwinter in the egg stage and hatch in early spring after their host tree produces new growth. In the fall, we have observed adults aggregating on goldenrod plants. In central Illinois, adults first appear in mid-June and become scarce by middle to late September. The adults emit a conspicuous odorous secretion when disturbed.

Interestingly, the black locust bug appears to be involved in a mimicry relationship with the adult stage of the locust leaf miner beetle *Odontota dorsalis* (Thurnburg) (Chrysomelidae). The larvae feed on black locust and the adult beetles closely resemble the black locust bug in color, pattern, and size. Additionally, both species occur at the same time of year and can be found together on the same plant where their ranges overlap.

In this paper, we identify the volatile chemical components and glandular source of the secretion in the black locust bug. Additionally, we examine the palatability of *L. robiniae* against various avian predators and provide evidence that the bugs are chemically defended and that the secretion functions as a feeding deterrent against predators.

METHODS AND MATERIALS

Insect Collection and Care

Adult black locust bugs, *Lopidea robiniae* (Uhler), were collected on black locust trees, *Robinia pseudoacacia* L., in and near Normal, Illinois, USA, from late July to mid-September 1998. Thereafter, the bugs were maintained until

needed in 2-liter translucent plastic jars at 25°C under a 14L:10D photoperiod and fed daily with fresh cuttings of black locust twigs and leaves, with the base of the twig inserted into a vial of water. Black locust bugs readily emit their secretions in response to even mild disturbances (see below). Therefore, during collecting, maintaining, and testing, the bugs were handled in an extremely gentle manner.

Chemical Analysis

Twenty *L. robiniae* adults were separated by sex using a dissecting microscope, then placed individually into glass GC autosampler vials (12 × 32 mm; Hewlett-Packard) and sealed with a Teflon-lined aluminum crimp cap. Before sealing the vials, the adults therein were agitated by gently poking them with a probe. Undisturbed male and female insects were also individually isolated in vials to serve as controls. We used solid-phase microextraction (SPME) (100 μm polydimethylsiloxane fiber; Supelco Inc., Bellefonte, Pennsylvania) to sample volatiles from individual vials. Sampling time was 30–45 min. Volatiles obtained from SPMEs of individual bugs were analyzed using a gas chromatograph (GC) (Hewlett-Packard model 5890 Series II) equipped with split/splitless injector run in splitless mode. The injector port was purged after 30 sec. Compounds were separated using a 30-m × 0.25-μm ID DB-5 column (J & W Scientific, Folsom, California) with a temperature program of 50°C for 1 min, followed by a ramp of 50°C to 250°C at 10°C/min, and remaining at 250°C for 6 min. Individual compounds were detected by flame ionization.

Mass spectra were obtained with a Hewlett-Packard 5973 MSD mass spectrometer. We introduced samples through a DB-SMS capillary column (30 m × 0.25 mm ID, 1.0-μm film) using a temperature program of 50°C for 1 min, followed by a ramp of 50°C to 250°C at 10°C/min, and remaining at 250°C for 6 min.

Authentic standards of (*E*)-2-hexenal (99%), (*Z*)-2-hexenal (97%), (*E*)-2-hexen-1-ol (96%), (*Z*)-2-hexen-1-ol (95%), (*E*)-3-hexen-1-ol (98%), (*Z*)-3-hexen-1-ol, (98%) (*E*)-2-heptenal (99%), (*Z*)-2-heptenal (97%), (*Z*)-3-octen-1-ol (94%), (*E*)-2-octenal (99%), (*Z*)-2-octenal (97%), (*E*)-2-octen-1-ol (96%), and (*Z*)-2-octen-1-ol (95%) were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Relative concentrations of individual secretion components were determined from GC peak areas, after compensating for biases for alcohols and heavier compounds inherent in SPME sampling (Bartelt, 1997).

Behavioral Response toward Threats

We used a 3-mm-diam. wooden dowel to examine the behavioral response of *L. robiniae* to threats. Ten wild adults were individually tested in the field on a warm sunny day (33–35°C) by moving the tip of the dowel several times to within

0.5 cm of the insect, at a rate of 2 cm/sec, then catching the insects in a net, and finally, holding the insects between the fingers.

Predator Feeding Trials

We tested six bird species for their ability to feed on black locust bugs. Birds were obtained as nestlings and raised in the laboratory on a diet of various arthropods and cat food supplemented with bird vitamins, earthworms, fruit, snails, and small fish. The specific diet approximated the natural diet of each bird species. In all cases, birds were tested only after they had developed the ability to feed independently and discriminate among food items. All the birds had experienced a variety of both palatable and chemically defended prey in the week prior to testing. For the robin (*Turdus migratorious*), brown thrasher (*Toxostoma rufum*), and blue jay (*Cyanocitta cristata*), this included several encounters with black locust bugs; hence, these birds had already experienced *L. robiniae*. In contrast, the killdeer (*Charadrius vociferus*), starling (*Sturnus vulgaris*), and the house wrens (*Troglodytes aedon*) had never encountered black locust bugs when tested and hence were naive vis-à-vis this insect.

The birds were starved for 2–4 hr prior to testing, and each was tested independently in 0.3- to 1.0-m^3 cages. Generally, each bird was offered a series of live *L. robiniae* adults until the bird refused to attack further offerings. Then the bird was offered alternative live palatable arthropods (crickets, *Acheta domesticus* L., and/or mealworms, *Tenebrio molitor* L.) of approximately the same mass as the black locust bugs. In some trials, birds received bugs and palatable prey in alternation. In addition to these tests using solitary birds, we also introduced bugs into a 1-m^3 cage containing a group of six wrens. Each test was completed within 48 min. After the trials, birds were released in appropriate habitats.

Blue Jay, Brown Thrasher, and American Robin. During the trials, each bird was offered a series of live *L. robiniae* by placing individual bugs in a Petri dish in the cage until the bird rejected at least four in a succession. Then, each was offered a series of five mealworms and three crickets.

Killdeer. The killdeer was first given a control third-instar *A. domesticus* cricket to demonstrate hunger. Then, individual *L. robiniae* were offered successively until the bird no longer consumed the bugs. When this occurred, the bird was then offered another control cricket to verify that the bird was not simply satiated. If the control was eaten, another treatment was then offered. Uneaten bugs were removed 4 min after placing them in the cage with the bird. This protocol was continued until the bird repeatedly ate the control insects, but repeatedly rejected the bugs, at which time the experiment was ended.

European Starling. This bird was tested with a protocol identical to that of the killdeer (above) except that mealworms were used as controls. With this predator,

we also recorded time to attack (the time from introduction of each insect until the bird pecked at the insect).

House Wren. A Petri dish containing seven live black locust bugs was placed into the bird's cage. Ten minutes later (when the wren had stopped attacking the bugs), a second Petri dish was added that contained four black locust bugs, four small crickets, and one mealworm. Eleven minutes later a third Petri dish containing five black locust bugs was added.

In a second experiment, we tested six wrens held communally in a 1-m^3 cage. A Petri dish containing 15 live black locust bugs was placed on the floor of the cage. Nine minutes later we added a Petri dish containing five crickets and four mealworms. Fourteen minutes later, we added a third dish containing 10 crickets and 10 black locust bugs.

Source of Volatiles

We examined *L. robiniae* adults under a dissecting microscope to determine the presence and location of external gland orifices. We then tested four locations as the possible source of the bugs' odorous secretion: mouth, anus, MTG, and DAG. Two adult males and two adult females were chilled to 10°C. One of each sex was glued ventral side up onto cardstock. The remaining two adults were dewinged using small scissors and glued ventral side down. All bugs were then allowed to warm to room temperature, viewed under a dissecting microscope, and poked or pinched with tweezers to elicit expulsion of the secretion. Slivers of filter paper (ca. 3.0 mm^2) were then placed on the MTG opening, dorsal abdomen, beak, or anus to absorb any ejected fluids, which were then examined for the presence or absence of odor.

RESULTS

Chemical Analysis. No volatiles were obtained from SPME of nonagitated adult male or female *L. robiniae*. Agitated bugs released a blend of at least six different volatiles (Figure 1) that were tentatively identified by matching mass spectra to library spectra. Chemical identification of five of these compounds was confirmed by comparing mass spectra and GC retention times (Table 1, Figure 2) to commercial standards. The sixth compound (peak 4) was identified as (*Z*)-3-octen-1-ol. It matched the authentic standard with respect to both GC retention time and mass spectrum. An authentic standard of (*E*)-3-octen-1-ol was not available for comparison, but by analogy to the hexen-1-ols, the (*E*)- and (*Z*)-3-octen-1-ols should be easily separable by GC, with the *E* isomer eluting first. There was no evidence for the *E* isomer from the bugs.

The relative concentrations of the six identified volatiles were not significantly different between males and females (six separate two-sample *t* tests,

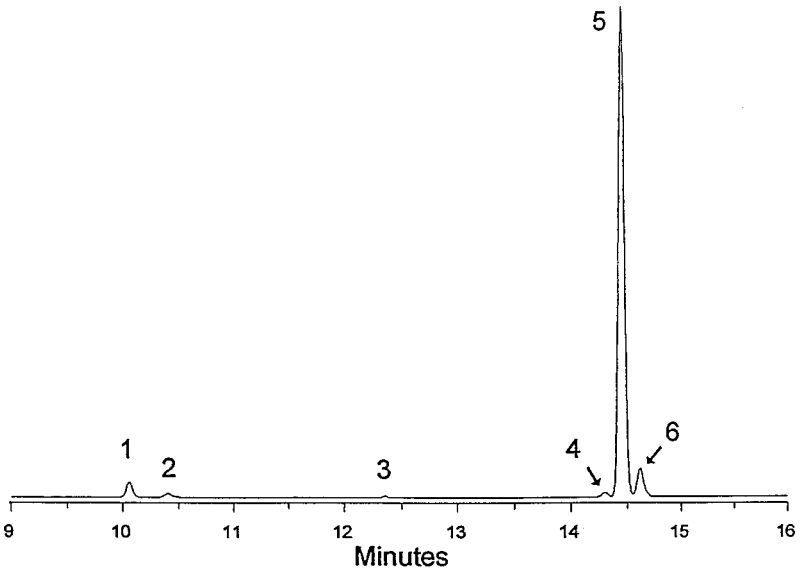


FIG. 1. Gas chromatogram of the metathoracic gland volatiles from a male black locust bug, *Lopidea robiniae*.

each $P > 0.05$). Averaged over both sexes ($N = 12$), the relative mean percentages and standard errors for the six compounds identified were: (*E*)-2-octenal, 75.5% ± 3.3; (*E*)-2-octen-1-ol, 15.5% ± 2.5; (*E*)-2-hexenal, 5.6% ± 1.8; (*E*)-2-hexen-1-ol, 2.0% ± 0.8; (*Z*)-3-octenol, 1.2% ± 0.3; (*E*)-2-heptenal, 0.2% ± 0.06.

TABLE 1. COMPOUNDS IDENTIFIED FROM *L. robiniae* (BLACK LOCUST BUG) SECRETIONS AND MS IONS

GC peak[a]	Identification	Major MS ions (percentages)
1	(*E*)-2-Hexenal	98 (M^+, 26), 83 (61), 70 (23), 69 (76), 57 (48), 55 (86), 42 (60), 41 (100)
2	(*E*)-2-Hexen-1-ol	100 (M^+, 2), 82 (22), 71 (11), 67 (18), 57 (100), 44 (17), 41 (40)
3	(*E*)-2-Heptenal	112 (M^+, 6), 97 (12), 83 (92), 70 (46), 69 (44), 68 (42), 57 (60), 56 (55), 55 (85), 43, (38), 41 (100)
4	(*Z*)-3-Octen-1-ol	128 (M^+, 1), 110 (14), 95 (15), 81 (63), 68 (53), 67 (43), 55 (100), 41 (51)
5	(*E*)-2-Octenal	126 (M^+, 0.4), 125 (0.9), 108 (4), 97 (14), 83 (61), 70 (90), 69 (46), 57 (58), 55 (100), 42 (42), 41 (100)
6	(*E*)-2-Octen-1-ol	128 (M^+, 1), 110 (8), 95 (12), 85 (8), 82 (24), 81 (29), 68 (31), 67 (26), 57 (100), 55 (47), 43 (41), 41 (56)

[a] Peak numbers correspond to those in Figure 1.

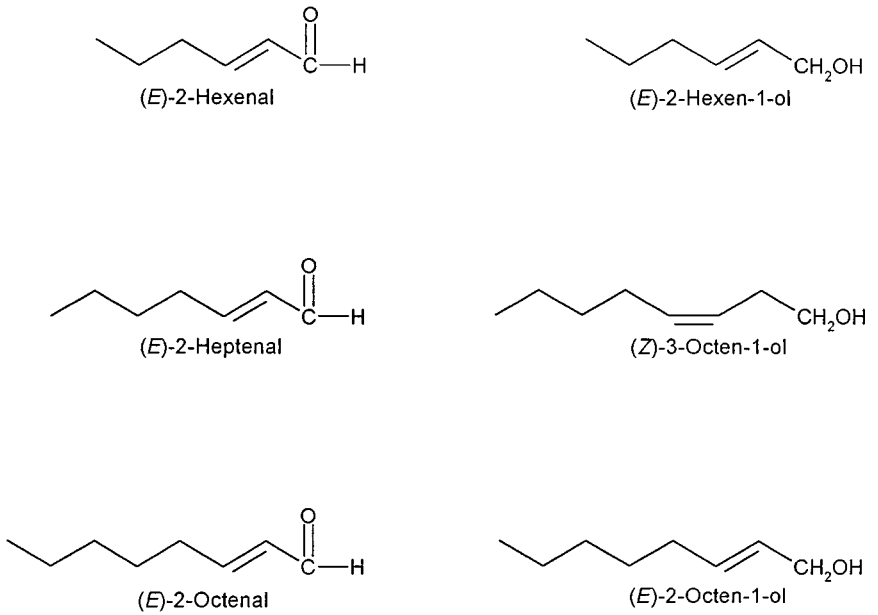


FIG. 2. Compounds identified in *Lopidea robiniae* (black locust bug) male and female metathoracic gland secretions.

Several other minor compounds were noted during GC-MS analysis, but were not identified.

Behavioral Response towards Threats. Nine of 10 adult *L. robiniae* showed defensive behavior when approaching dowels were about 6.0 cm away by quickly crawling away from the dowel to the opposite side of the leaf or stem. Pursuing the bug further with the dowel elicited escape by flight (7 of 10) or dropping (3 of 10), but failed to cause them to expel perceivable volatiles. Four of the 10 bugs secreted when simply caught in the insect net. Five of the remaining six bugs secreted when held between the fingers.

Predator Feeding Trials. All six species of bird tested showed some form of aversion to feeding on black locust bugs, although the exact response varied among individual birds (Table 2). The three birds that had previously encountered black locust bugs (jay, thrasher, robin) consumed only 2 of 15 bugs offered, yet ate 23 of 24 palatable crickets and mealworms. The killdeer and starling, which had not previously experienced *L. robiniae*, initially consumed the bugs, then abruptly stopped and refused all subsequent bugs. The killdeer vomited and the starling gagged (see below). The solitary house wren was also naive yet refused to eat a single black locust bug. Bugs that were attacked by birds emitted their secretion, producing a distinct odor in and near the cage.

TABLE 2. FEEDING RESPONSE OF SIX BIRDS TO BLACK LOCUST BUGS (*Lopidea robiniae*), CRICKETS (*Acheta domesticus*), AND MEALWORMS (*Tenebrio molitor*)

Predator	Bugs (*N*)		Mealworms (*N*)		Crickets (*N*)	
	Offered	Consumed	Offered	Consumed	Offered	Consumed
Blue Jay[a]	5	1	5	5	3	3
Thrasher[a]	5	1	5	5	3	3
American robin	5	0	5	5	3	2
Killdeer[b]	22	12	—	—	10	10
European starling[c]	17	12	8	8	—	—
House wren	16	0	1	1	4	2

[a] Only the first bug encountered was consumed. All subsequent bugs were rejected.
[b] The killdeer ate the first 12 bugs offered, then vomited. Afterwards, it refused all subsequent bugs offered, but ate all crickets offered.
[c] The starling gagged after consuming 12 bugs and 6 mealworms.

The blue jay attacked and swallowed the first bug 46 sec after it was introduced. One minute later we introduced a second bug, which was immediately seized and quickly flung from the beak. At 3 min from the start of the trial, a third bug was offered and was likewise seized and ejected. At 4 min, a fourth bug was introduced; 32 sec later the jay bit and dropped this insect. At 6 min, a fifth bug was introduced. For 5 min, the jay ignored this bug as well as the three previously ejected bugs, which remained in the cage. At 11 min, five mealworms and three crickets were introduced at 1-min intervals. The jay immediately attacked and consumed each, but continued to ignore the bugs remaining in the cage.

The thrasher attacked and swallowed the first bug 3 sec after it was offered. One minute later, a second bug was offered. It was seized within 2 sec and immediately flung from the mouth. Five seconds later the bird picked up the bug again, held it in its mouth for 4 sec and dropped it. At 2 min, a third bug was offered and was immediately seized and dropped. At 3 min, a fourth bug was offered. The thrasher landed next to the bug as if to attack, but turned and flew away. At 4 min, a fifth bug was introduced. The thrasher again landed near the bug, lowered its head as if to attack, then flew away. At 10 min, five mealworms and three crickets were offered at 1-min intervals; all were immediately attacked and consumed. The bird continued to ignore the remaining black locust bugs.

The robin immediately attacked and dropped the first bug offered. During the next 40 sec, it seized and dropped the bug three additional times. At 2 min from the beginning of the trial, a second bug was introduced and was ignored by the robin. At 3 min, a third bug was introduced, and it was immediately seized and dropped. At 4 and 5 min, a fourth and fifth bug were introduced and were ignored. At 10 min, five mealworms and three crickets were introduced at 1-min intervals. The robin quickly consumed the mealworms and two crickets, but continued to ignore the bugs remaining in the cage.

The killdeer ate the first control cricket, demonstrating that it was hungry. The bird was then offered 12 bugs in a row, but was hesitant to eat the first four, which were not eaten until 35–145 sec after they were introduced. However, the next eight were readily attacked and consumed, and each bug expelled its secretion, producing a strong odor. The thirteenth bug offered was rejected, and the second control was then subsequently offered and eaten immediately by the bird. The fourteenth bug was also rejected. The bird then vomited (after consuming 2 crickets and 12 bugs). Thereafter, the bird rejected all subsequent bugs (Table 2), and would (1) bob its head toward its prey as if to attack and then quickly withdraw without touching the prey, (2) actively pace around the bug, or (3) completely ignore the bugs. When a bug flew onto the killdeer's back, it quickly turned its head, bit and released the bug (without harming it), and then began biting the paper on the bottom of the cage. In contrast, the bird immediately consumed all 10 control crickets and, on one occasion, the bird stepped past three moving bugs to get to a cricket.

The starling demonstrated mild aversion towards adult *L. robiniae*. It consumed 12/17 bugs offered vs. 8/8 mealworms (Table 2), but the time to attack was significantly longer for *L. robiniae* (190.9 ± 21.0 sec) than for mealworms (2.3 ± 0.3 sec) (two sample *t* test, $P < 0.001$; $df = 7$). During the trial, the starling showed aversive behavior toward *L. robiniae*, such as ejecting bugs out of the beak and running toward newly introduced bugs, staring at them from 2 cm away, and then turning away. Near the end of the trial, the bird exhibited a gagging reflex by opening its mouth wide and thrusting its head and neck forward. It then closed its beak and swallowed, which suggests the bird was nauseated and possibly vomited into its esophagus or mouth.

The solitary wren attacked four bugs in quick succession, immediately flinging each bug away. During the next 4 min, it inspected bugs without attacking them. Four minutes into the test, the wren picked up and dropped a bug, then immediately wiped its beak on the floor of the cage. At 10 min, a second Petri dish was added, that contained four small crickets, four black locust bugs, and one mealworm. Within 15 sec, the wren ate two crickets and the mealworm. At 21 min, a third Petri dish was introduced that contained five live black locust bugs. The wren inspected these bugs, but did not attack.

When 15 black locust bugs were placed in a cage containing six house wrens, all birds quickly approached and attacked the bugs. By 5 min after the start of the test, 23 attacks (=bites) had occurred. In 19 of these attacks, the bug was rejected; however, one individual bird consumed four bugs. At 9 min, five small crickets and four mealworms were added to the cage. By 14 min, two mealworms, five crickets, and one of the remaining black locust bugs were eaten. At 23 min, 10 small crickets and 10 black locust bugs were added. By 24 min, nine crickets but no black locust bugs had been eaten. At 27 min, the experiment was concluded with a total consumption of 5 bugs, 2 mealworm, and 14 crickets.

Source of Volatiles. When adult male and female *L. robiniae* were disturbed, an odorous liquid emerged from the MTG openings suggesting that the MTG was the source of the scent. In contrast, filter paper slivers placed on the mouth, anus, or dorsal abdomen of disturbed adults acquired no scent.

DISCUSSION

Chemistry of Lopidea robiniae Secretion. We identified three unsaturated alcohols and three unsaturated aldehydes in the defensive secretion of adult *L. robiniae*. True bugs commonly produce six- and eight-carbon unsaturated (*E*)-alkanes and (*E*)-alcohols (Blum, 1981; Aldrich, 1988; McBrien and Millar, 1999). Hence, the compounds identified in *L. robiniae* represent, for the most part, typical hemipteran exocrine products. Four of the six identified compounds, [(*E*)-2-hexenal, (*E*)-2-hexen-1-ol, (*E*)-2-octenal, and (*E*)-2-octen-1-ol] are common in insect exudates. (*E*)-2-Hexenal is found in the defensive secretions of ants (Crewe et al., 1972), beetles (Tschinkel, 1975), cockroaches (Farine et al., 1997), and in true bugs (Percy et al., 1980; Knight et al., 1984; Hamilton et al., 1985; Staddon et al., 1987; Aldrich, 1988; Borges and Aldrich, 1992; Farine et al., 1992a,b; Leal et al., 1994; Krall et al., 1999; McBrien and Millar, 1999). (*E*)-2-Hexen-1-ol has similarly been identified in the defensive secretions of ants (Crewe et al., 1972), cockroaches (Wallbank and Waterhouse, 1970), and in the pheromone or defensive secretions of numerous Heteroptera (Hamilton et al., 1985; Aldrich, 1988; Smith et al., 1991; Farine et al., 1992a,b; McBrien and Millar, 1999). (*E*)-2-Octenal has been identified in cockroaches (Wallbank and Waterhouse, 1970; Farine et al., 1997), and numerous species of Heteroptera (Percy et al., 1980, Blum, 1981; Knight et al., 1984; Hamilton et al., 1985; Staddon et al., 1987; Aldrich, 1988; Borges and Aldrich, 1992; Farine et al., 1992a,b; Aldrich et al., 1995; Leal et al., 1996; Krall et al., 1997; McBrien and Millar, 1999). (*E*)-2-Octen-1-ol has been found in cockroaches (Wallbank and Waterhouse, 1970; Farine et al., 1997), sawflies (Boeve et al., 1997), and several Heteroptera (Aldrich and Yonke, 1975; Percy et al., 1980; Staddon et al., 1987; Farine et al., 1992a,b; Aldrich et al., 1993; Leal et al., 1996; Krall et al., 1997). (*E*)-2-Heptenal occurs less frequently in insects, relative to the other structurally similar chemicals identified in our research, and has been reported in at least one tenebrionid beetle, *Eleodes beamri* (Tschinkel, 1975) and in the heteropteran families Pentatomidae (Blum et al., 1960) and Cydnidae (Roth, 1961). Trace amounts of both stereoisomers of 3-octen-1-ol (e.g., $<1\%$ total secretion) have been found in cockroaches (Farine et al., 1997); however, this compound appears to be exceedingly rare among terrestrial arthropods and, to our knowledge, has never been reported as a primary constituent of any insect defensive secretion.

It is interesting to note that four of the compounds identified in this research are frequently found in a mixture in other Heteroptera. For example,

(*E*)-2-hexenal, (*E*)-2-hexen-1-ol, (*E*)-2-octenal, and (*E*)-2-octen-1-ol are present in the pheromone/defensive secretions of two members of Pyrrhocoridae (Farine et al., 1992a,b). (*E*)-2-Hexenal, (*E*)-2-octenal, and (*E*)-2-hexen-1-ol, are found in the pentatomid *Acrosternum pennsylvanicum* (Aldrich et al., 1995) and are part of a complex defensive secretion in the scutellerid bug *Hotea gambiae* that functioned to deter conspecific competitors and oviposition by parasitoids (Hamilton et al., 1985). Likewise, the former two aldehydes are extremely common in pentatomids (Aldrich et al., 1995; McBrien et al., 2001; Ho and Millar, 2001). The frequent cooccurrence of these compounds suggests a common enzymatic origin, and Blum (1981) has suggested that the alcohols may simply represent precursors to their aldehyde analogues.

Function of L. robiniae Secretion. Our results suggest that *L. robiniae* is chemically defended and that the volatiles found in the MTG secretion of *L. robiniae* function in antipredator defense. We base these conclusions on the following: (1) The secretion is discharged in direct response to threats. (2) Both sexes produce the same major components in the secretion, suggesting that they do not function as sexual pheromones. (3) Adults are brightly colored, implying an aposematic warning function (Pasteels et al., 1983; Guilford, 1990). (4) Adult black locust bugs occasionally aggregate, a trait often associated with chemically defended insects (Pasteels et al., 1983). (5) The types of compounds identified in the MTG secretion (short-chain aldehydes and alcohols) are volatile, highly odorous, and are known to have toxic or irritating effects (Eisner, 1970; Hamilton et al., 1985; Griffin and Segall, 1989; van Iersal et al., 1997). Hence, they are ideally suited to serve as alerting or punishing substances (Blum, 1981; Whitman et al., 1990) and can effectively deter predators (Blum, 1981; Hamilton et al., 1985; Aldrich, 1988; Gunawardena and Herath, 1991; Gunawardena and Bandumathie 1993; Krall et al., 1999). Finally, (6) Several predators tested in this study demonstrated feeding aversions towards *L. robiniae*. For example, after sampling a single bug, the jay and the thrasher rejected all others. The robin and several wrens refused to eat a single bug. Two birds exhibited symptoms of toxicosis after consuming black locust bugs: the killdeer vomited and the starling gagged. The most telling response was seen in the robin and the solitary wren: both birds eagerly attacked the first bugs offered, but then immediately ejected the bugs from their beaks, suggesting that the secretion itself was unpleasant. After biting bugs, the solitary wren wiped its beak on the floor of the cage, and the killdeer bit paper. These behaviors suggest that the secretion was irritating or unpleasant and that the birds were attempting to remove the offending secretion from their mouths.

It is interesting that the bird predators we tested displayed inter- and intraspecific variation in response to black locust bugs; some birds refused to attack, some ate only one bug, and some consumed several bugs. Some bugs were killed during the attacks, whereas other bugs survived relatively unscathed. Such differences in predator response to a single prey species are frequently observed in predator–prey

interactions (Brower, 1984; Whitman et al., 1985; Krall et al., 1999). Variability in predator response and prey survival is important for the evolution and continued maintenance of antipredator defenses in prey. It suggests that even limited or low-impact defenses would have selective advantages against timid predators. It also suggests that even a well-defended prey species would continue to experience selective pressure from naive, more aggressive, or less sensitive predators.

Chemicals in defensive secretions can function in different ways (Whitman et al., 1990), and it remains to be determined exactly how the compounds identified in this research facilitate prey rejection by bird predators. Defensive chemicals can be noxious Class I compounds, which poison, harm, or irritate predators, or relatively innocuous Class II compounds, which typically are not toxic themselves but function as chemical warning signals for other toxic compounds (Brower, 1984). Our observations suggest that secretions from *L. robiniae* may work on both levels. For example, the secretion appeared to be an immediate repellent to the robin and the solitary wren, which repeatedly spit out bugs without consuming them. Alternatively, the killdeer seemed little affected by the "taste" of the bug and appeared to consume them (at first) with impunity. However, this predator later vomited, suggesting that the black locust bug may possess internal toxins in addition to a secretory defense. Indeed, many chemically defended insects, exclusively or in addition to the use of volatile secretions, employ slower-acting, high-molecular-weight internal toxins, which are often sequestered directly from toxic host plants (Duffey, 1977; Blum, 1981; Aldrich, 1988; Bowers and Farley, 1990; Aldrich et al., 1997; Aliabadi and Whitman, 2001). Black locust trees contain a number of potent toxins, including the phytotoxin robin, the glycoside robitin, the alkaloid robinine, and numerous phenolics (Lewis and Elvin-Lewis, 1977; Duke, 1985; Bruneton, 1999). The consumption of black locust bark, leaves, or seeds causes serious neurointenstinal reactions in humans (especially children) and livestock (Kingsbury, 1964; Tampion, 1977; Stevens, 1980; Duke, 1985; Harborne and Baxter, 1993). Several Heteroptera appear to sequester defensive compounds from host material (Aldrich, 1988; Aliabadi and Whitman, 2001), presumably for chemical defense. Possibly, black locust bugs, which are thought to feed almost exclusively on black locust, may sequester plant toxins for their defense. Hence, *L. robiniae*, like many other chemically defended insects, might posses a paired defense mechanisms: an initial defense that warns or deters, backed up by an internal defense that is the primary basis for their unpalatability.

Sequestration of host plant toxins might explain the apparent mimicry relationship between the black locust bug and the locust leaf miner. At present it is not known which species is the model or the mimic, if the relationship is Batesian or Mullerian, or even if a true mimicry relationship exists. However, it is possible that both species sequester defensive substances from their common host plant.

In addition to a primary defensive role, the MTG secretions of some true bugs also function as sexual, aggregation, alarm, or dispersal pheromones (Aldrich,

1994, 1996; McBrien and Millar, 1999). This is true in the Miridae, where sexual pheromones from the MTG gland are known for a number of species (Knight et al., 1984; Thislewood et al., 1989; Aldrich, 1988, 1996; Millar et al., 1997; McBrien and Millar, 1999). Although *L. robiniae* occasionally aggregate, it is not yet known if their MTG secretions exhibit pheromonal activity.

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SCREENING FOR EFFECTS OF PHYTOCHEMICAL VARIABILITY ON CYTOPLASMIC PROTEIN SYNTHESIS PATTERN OF CROP PLANTS

TERESA ROMERO-ROMERO, ANA LUISA ANAYA, and ROCIO CRUZ-ORTEGA*

Laboratorio de Alelopatía
Departamento de Ecología Funcional y Aplicada
Instituto de Ecología, UNAM
Circuito Exterior Universitario, Ciudad Universitaria, 04510 México D.F.

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Abstract—Crop plants have to cope with phytochemical variability along with other environmental stresses. Allelochemicals affect several cellular processes. We tested the effect of toxic aqueous leachates from *Sicyos deppei*, *Acacia sedillense*, *Sebastiania adenophora*, and *Lantana camara* on the radicle growth and cytoplasmic protein synthesis patterns of *Zea mays* (maize), *Phaseolus vulgaris* (bean), *Cucurbita pepo* (squash), and *Lycopersicon esculentum* (tomato). 2D-PAGE and gel scan densitometry analysis were used to detect differences in cytoplasmic root protein pattern expression. High-, medium-, and low-molecular-weight cytoplasmic proteins were affected by the different aqueous leachates. Crop plant responses were diverse, but in general, an increase in protein synthesis was observed in the treated roots. Maize was the least affected, but both the radicle growth and also the protein pattern of tomato were severely inhibited by all allelopathic plants. The changes observed in protein expression may indicate a biochemical alteration at the cellular level of the tested crop plants.

Key Words—Allelochemical stress, allelopathy, mode of action, *Sicyos deppei*, *Acacia sedillense*, *Sebastiania adenophora*, *Lantana camara*, *Zea mays*, *Cucurbita pepo*, *Phaseolus vulgaris*, *Lycopersicon esculentum*, protein synthesis, phytochemical variability.

INTRODUCTION

Secondary plant metabolites that mediate interactions among different organisms are known as allelochemicals. These metabolites are released into the environment

* To whom correspondence should be addressed. E-mail: rcruz@ifisiol.unam.mx

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by different routes and belong to several chemical groups, including terpenes, alkaloids, phenolics, and flavonoids (Larcher, 1995). Plants, as sessile organisms, encounter an array of biotic and abiotic environmental constraints such as phytotoxic compounds, which can affect different cellular processes on target organisms. Effects on physiological processes are well known (Einhellig, 1995) and include disruption of membrane permeability (Galindo et al., 1999), ion uptake (Yu and Matsui, 1997), inhibition of electron transport in both the photosynthesis and respiratory chains (Calera et al., 1995a; Rimando et al., 1998; Abrahim et al., 2000), alteration of enzymatic activity (Calera et al., 1995b; Friebe et al., 1997; Politycka, 1999; Romagni et al., 2000), and inhibition of cell division (Cruz-Ortega et al., 1988; Anaya and Pelayo-Benavides, 1997).

Environmental stresses (biotic and abiotic) have also been shown to induce the synthesis of new proteins in plants. These proteins might have evolutionary value for survival under adverse environmental situations. Synthesis of such stress-induced proteins has been well documented under stress by salinity, drought, heat shock, cold, anaerobiosis, metal toxicity, and pathogenicity (Cruz-Ortega et al., 1997; Riccardi et al., 1998; Seki, 2001), but there are few reports about allelochemical stress (Baziramakenga et al., 1997; Koitabashi et al., 1997).

The plants from El Eden Ecological Reserve (Quintana Roo, Mexico) tested in this study were previously evaluated in our laboratory for their phytotoxic effects on other plants (Anaya and del Amo, 1999). Currently, we are conducting biodirected fractionation studies to determine the chemical nature of the active metabolites. As a part of this project, we screened the changes induced in root cytoplasmic protein synthesis pattern of maize, bean, squash, and tomato by the aqueous leachates, with the aim of contributing to the knowledge of the modes of action of the stress produced by the phytotoxic compounds.

METHODS AND MATERIALS

Phytotoxic Plants. Leaves from *Acacia sedillense* L. Rico (Fabaceae), *Sebastiania adenophora* Pax & K. Hoffm. (Euphorbiaceae), and *Lantana camara L*. (Verbenaceae) were collected at El Eden Ecological Reserve in Quintana Roo, Mexico. *Sicyos deppei* G. Don (Cucurbitaceae) leaves were collected in some crop fields in the Mexico basin.

Plant Material. Seeds of maize (*Zea mays* L., cv. Chinampero, Poaceae), squash (*Cucurbita pepo* L. cv. Criolla, Cucurbitaceae), and bean (*Phaseolus vulgaris* L. var. Flor de mayo, Fabaceae) were obtained from a local market at Tulyehualco, Mexico, D. F. Tomato seeds (*Lycopersicon esculentum,* var. Pomodoro, Solanaceae) were obtained from Sun-Seeds, Parma, Idaho, USA.

Bioassays. Seeds were germinated in Petri dishes containing aqueous leachate (1% w/v) of each phytotoxic plant with 1% agar as substrate. Aqueous leachates were prepared by soaking dried leaves (2 g/100 ml) in distilled water for 3 hr

and were mixed with agar (2%) for a final aqueous leachate concentration of 1%. Leachates were filtered through Whatman No. 4 paper and then through a Millipore membrane (0.45 μm). Osmotic pressure was measured by using a freezing-point osmometer (Osmette A, Precision System, Inc.); all values ranged from 15 to 17 mosm/liter. Bioassays were conducted under sterile conditions in a laminar hood. Controls contained only distilled water and agar. Ten seeds were placed on each Petri dish and kept in the dark at 27°C. Twenty-five Petri dishes were used per treatment and per crop plant. The terminal 0.5 cm of the primary root was excised after 48 hr from maize, squash, and beans seedlings and after 72-hr from tomato seedlings. Roots were frozen in liquid nitrogen and kept at −70°C until use. For root growth response, experiments were conducted in a complete randomized experiment design with four replicates. Primary root lengths were measured after 48 and 72 hr, and data were analyzed by ANOVA.

Cytoplasmic Protein Extraction, Gel Electrophoresis, and Densitometry Analysis. Cytoplasmic proteins were extracted and purified from roots of control and treated plants. The terminal 0.5 cm of the primary root from 300 treated and control seedlings was homogenized in liquid nitrogen with a mortar and pestle and suspended (1:4 w/v ratio) in a cold homogenization buffer [50 mM K_2HPO_4 and 50 mM of KH_2PO_4, pH 6.8, 1 mM phenylmethylsulphonyl fluoride (PMSF)]. The homogenate was centrifuged at 300*g* for 10 min to pellet the nuclear fraction, and the supernatant was centrifuged at 12,000*g* for 10 min at 4°C. Proteins from the supernatant were extracted into phenol, precipitated with methanol, and redissolved in an isoelectric focusing (IEF) medium (ampholyte solution at pH 4–7) as described by Hurkman and Tanaka (1988). Protein content was determined by the Bradford method (1976).

Two-dimensional gel electrophoresis (2D-PAGE) was performed according to O'Farrell (1975). For the first dimension, 10 μg of protein was loaded at the basic end of the gels (capillary tubes, Bio-Rad). Isoelectric focusing was conducted for 30 min at 300 V, and then for 4 hr at 750 V. After extrusion, gels were either frozen at −70°C or loaded onto a second dimension of 12% polyacrylamide resolving gel. Gels were run in a mini-protein apparatus (Bio-Rad), fixed, and silver stained according to Morrisey (1981). Controls and treated gels were analyzed by GelScan XL (release 2.1) densitometry. Total area and absorbance of protein spots on the gels were determined, adjusted for background, and assessed for any change in size.

RESULTS

Aqueous leachates (1%) of the four phytotoxic plants affected maize (*Zea mays*) radicle growth differently, with *S. deppei* leachate causing 39% ($P < 0.001$) growth inhibition, while *A. sedillense*, *S. adenophora*, and *L. camara* had no

TABLE 1. RADICLE GROWTH AND CYTOPLASMIC PROTEINS ALTERED IN *Zea mays*, *Cucurbita pepo*, *Phaseolus vulgaris*, AND *Lycopersicon esculentum* BY AQUEOUS LEACHATES (1%) OF *Acacia sedillense*, *Sebastiania adenophora*, *Lantana camara*, AND *Sicyos deppei*

Crop plant	Allelochemical plant	Radicle growth (%)	Proteins (Number)				
			Increased	Decreased	Repressed	Induced	Total
Z. mays	*A. sedillense*	112	18	1	2		21
	S. adenophora	115	9	2		3	14
	L. camara	107	3	7			10
	S. deppei	61[a]					0
C. pepo	*A. sedillense*	80	16	3			19
	S. adenophora	149[a]	4	5			9
	L. camara	70[a]	12	4	2		18
	S. deppei	104	6	4	3		13
P. vulgaris	*A. sedillense*	75[a]	16				16
	S. adenophora	83	7	3			10
	L. camara	59[a]	5	9		1	15
	S. deppei	30[a]					0
L. esculentum	*A. sedillense*	40[a]	12		1	1	14
	S. adenophora	30[a]	4	6	1		11
	L. camara	19[a]	6	5			11
	S. deppei	19[a]	4	6	1		11

[a] $P < 0.001$.

significant effect (Table 1). Although the leachate of *S. deppei* inhibited maize root growth, no change was observed in cytoplasmic protein synthesis (results not shown). In contrast, *A. sedillense*, *S. adenophora*, and *L. camara* caused several changes in its cytoplasmic protein synthesis pattern (Figure 1). Twenty-one proteins were altered by *A. sedillense* leachate, with 18 increased (proteins 1–11, 14–18, 20, and 21), 1 decreased (protein 19), and 2 repressed (proteins 12 and 13). *S. adenophora* leachate affected 14 proteins, with 9 increased (proteins 1, 2, and 6–12), 2 decreased (13 and 14), and 3 induced (3–5). Ten proteins were altered by *L. camara*, with 7 decreased (proteins 1, 3, and 6–10) and only 3 increased (2, 4, and 5) (Table 1, Figure 1). These results indicate that maize responds differently to various phytochemical stresses caused by different leachates. Analysis of cytoplasmic proteins revealed that different aqueous leachates affected the same proteins differently (Table 2). For example, a protein with apparent molecular weight of 57 kDa, and pI of 5.5 was repressed with *A. sedillense* leachate but decreased with *L. camara*. Another protein (27 kDa, pI 6.4) increased with *A. sedillense*, *S. adenophora*, and *L. camara* leachates. Three low-molecular-weight proteins, 18 (pI 6.1), 16 (pI 5.8), and 15 (pI 5.7) kDa, were induced only with *S. adenophora* leachate (Table 1, Figure 1D, proteins 2–4).

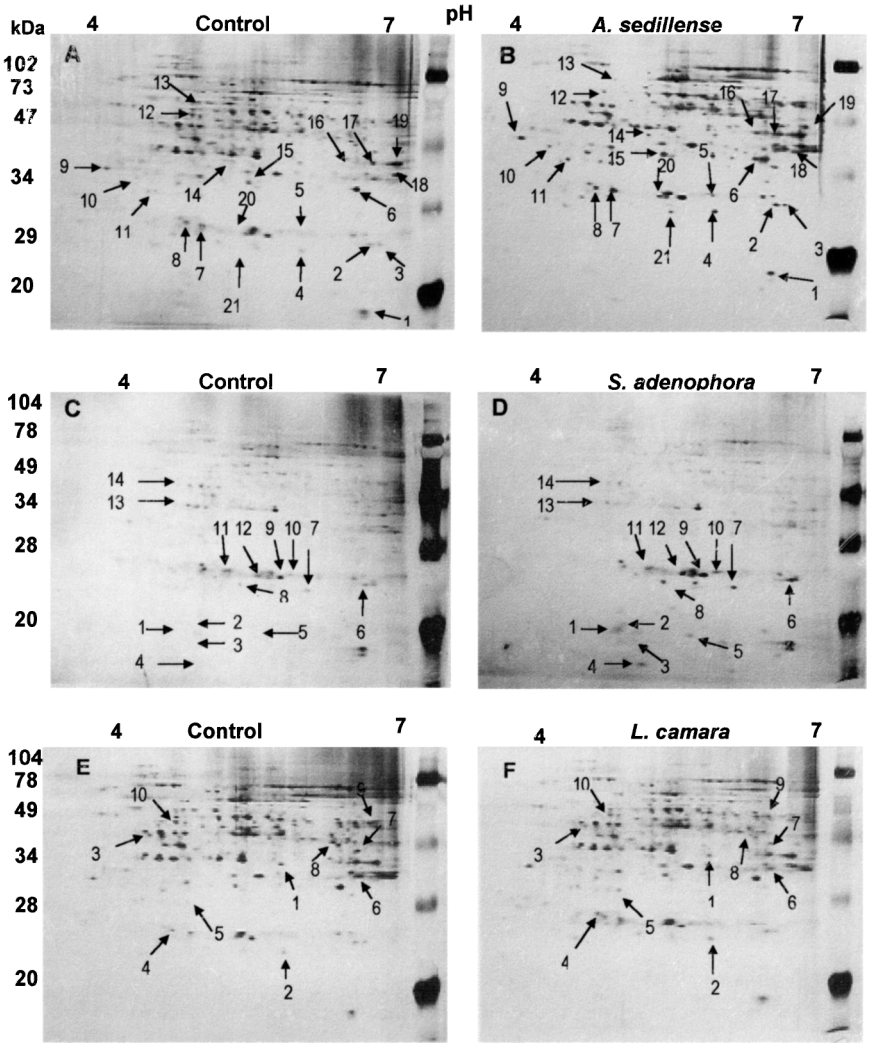


FIG. 1. 2D-PAGE of cytoplasmic root proteins from control maize seedlings (A, C, and E) and those subjected to the aqueous leachates: *A. sedillense* (B); *S. adenophora* (D), and *L. camara* (F). Ten micrograms of the proteins were loaded into each gel. Arrows indicate those proteins that were altered and detected by gel scanning. Gels represent the mean of at least three replicates. Molecular masses in kilodaltons (kDa) are indicated.

TABLE 2. PROTEINS OF SAME MOLECULAR WEIGHT AND pI ALTERED IN ROOTS OF *Zea mays* BY AQUEOUS LEACHATES OF *Acacia sedillense*, *Sebastiania adenophora*, AND *Lantana camara*[a]

Protein (kDa)	pI	*A. sedillense*	*S. adenophora*	*L. camara*
57.0	5.5	E		D
30.0	5.5	I		I
29.0	6.0	I	I	
28.0	6.8	I	I	
27.0	6.4	I	I	I

[a] I = increased; D = decreased; E = repressed.

Table 1 shows that *L. camara* inhibited squash (*Cucurbita pepo*) radicle growth by 30% ($P < 0.001$), while *S. adenophora* had a stimulatory effect (49%) ($P < 0.001$). No significant effect was observed with *A. sedillense* and *S. deppei* leachates. All four leachates also differently affected the protein synthesis in squash (Figure 2). *A. sedillense* and *L. camara* caused the most changes (a total of 19, and 18 proteins, respectively; Figure 2D, H, Table 1). Of the 19 proteins altered by *A. sedillense*, 16 increased (proteins 1, 4–8, and 10–19) and three decreased (2–4) (Table 1). Of the 18 altered by *L. camara*, 12 increased (6, and 8–18), 4 decreased (1, 3, 5, and 7), and 2 were repressed (2 and 4). In contrast, *S. adenophora* leachate, which significantly stimulated radicle growth, induced fewer changes in cytoplasmic proteins: 9 were altered with 4 increased (1–3, and 7) and 5 decreased (4–6, 8, and 9). *S. deppei* altered 13 proteins with 6 increased (5, 8–11, and 13), 4 decreased (3, 6, 7, and 12) and 3 repressed (1, 2, and 4) (Fig. 2b, F, Table 1). Table 3 shows that the same protein was differently affected by the different aqueous leachates. A16-kDa protein (pI 6.2) decreased with *S. deppei* and *L. camara* but increased with *A. sedillense*. The expression of a 15-kDa (pI 6.0) protein was repressed, increased, and decreased by *S. deppei*, *A. sedillense*, and *L. camara*, respectively.

As in maize and squash, aqueous leachates affected *P. vulgaris* radicle growth differently. *S. deppei*, *L. camara*, and *A. sedillense* leachates inhibited it 70%, 41%, and 25% ($P < 0.001$), respectively, while *S. adenophora* did not have any effect (Table 1). Although *S. deppei* strongly inhibited root growth in beans, it did not affect the expression of proteins. *A. sedillense* increased the expression of 16 proteins, and *L. camara* leachate altered 15 proteins: 5 proteins increased (2, 8–11), 9 decreased (3–7, 12–15), and one induced (1). *S. adenophora* altered 10 proteins, including 7 that increased (3, 5–10) and 3 that decreased (1, 2, and 4) (Table 1, Figure 3B, D, F). Two cytoplasmic proteins were differently affected by leachates. The expression of a 24-kDa (pI 5.8) protein was increased by *A. sedillense* but decreased by *S. adenophora*, while a 26-kDa (pI 5.9) protein was increased by both *A. sedillense* and *L. camara* (Figure 3B, F).

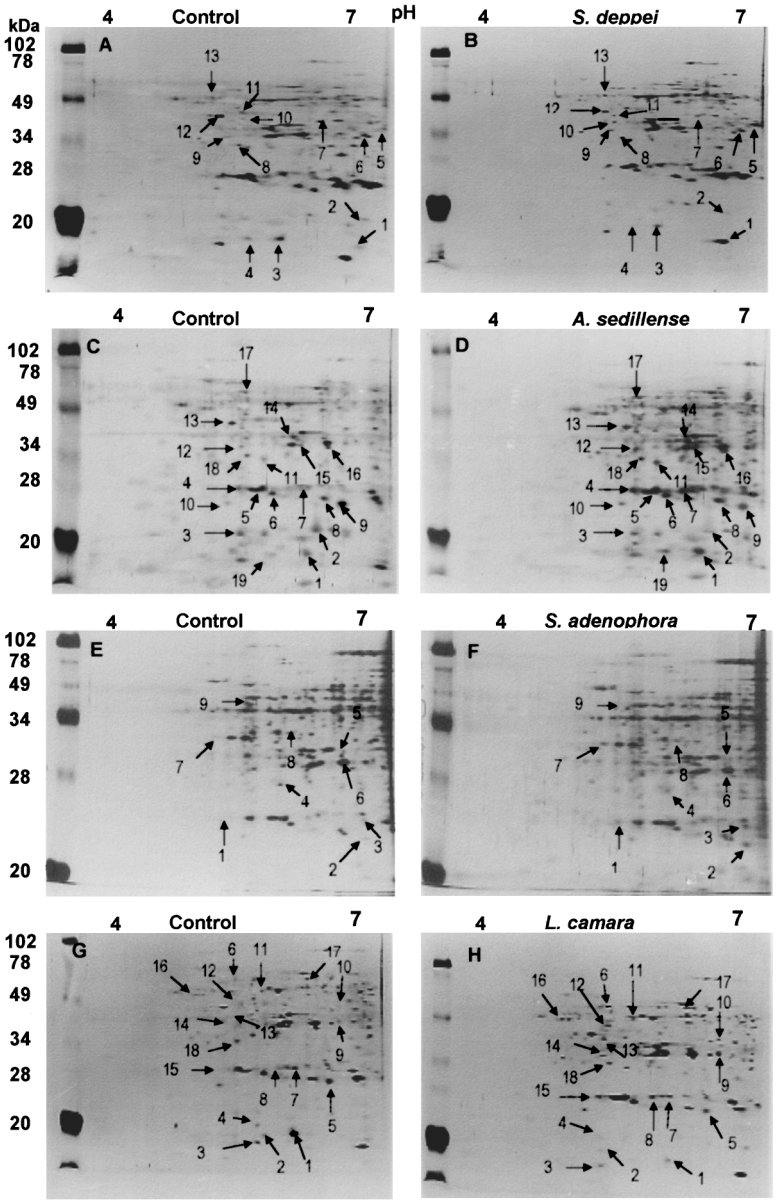


FIG. 2. 2D-PAGE of cytoplasmic root proteins from control squash seedlings (A, C, E, and G) and those subjected to the aqueous leachates: *S. deppei* (B), *A. sedillense* (D); *S. adenophora* (F); and *L. camara* (H). Ten micrograms of the proteins were loaded in each gel. Arrows indicate those proteins that were altered and detected by gel scanning. Gels represent the mean of at least three replicates. Molecular masses in kilodaltons (kDa) are indicated.

TABLE 3. PROTEINS OF SAME MOLECULAR WEIGHT AND pI ALTERED IN ROOTS OF *Cucurbita pepo* BY AQUEOUS LEACHATES OF *Sicyos deppei*, *Acacia sedillense*, *Sebastiania adenophora*, AND *Lantana camara*[a]

Protein (kDa)	pI	*S. deppei*	*A. sedillense*	*S. adenophora*	*L. camara*
63.0	6.7		I		I
49.0	5.8	D	I		
47.0	5.9	I			I
40.0	5.9	I	I		I
39.0	6.0	I	I		I
27.0	5.7		I		I
25.0	6.0		I		I
22.0	5.7		D	I	D
16.0	6.2	D	I		D
15.0	6.0	E	I		D

[a] I = increased; D = decreased; E = repressed.

Tomato (*Lycopersicon esculentum*) seedlings were the most sensitive to phytochemical stress. All aqueous leachates significantly ($P < 0.001$) inhibited radicle growth, ranging from 81% for *S. deppei* and *L. camara*, to 70% for *S. adenophora* and 60% for *A. sedillense* (Table 1). The aqueous leachates also similarly affected the number of protein expressed in tomato, with a total of 11 each for *S. deppei*, *S. adenophora*, and *L. camara*; and 14 for *A. sedillense* (Table 1, Figure 4), with nearly an equal number of proteins increasing and decreasing for each leachate. Eight proteins were differently affected by leachates in tomato. For example, a high-molecular-weight protein (70 kDa, pI 5.5) was repressed by *A. sedillense* but was decreased by *L. camara*. A 34-kDa (pI 6.9) protein decreased under *S. deppei* but was increased by *A. sedillense* and *S. adenophora* (Table 4, Figure 4).

TABLE 4. PROTEINS OF SAME MOLECULAR WEIGHT AND pI ALTERED IN ROOTS OF *Lycopersicon esculentum* BY AQUEOUS LEACHATES OF *Sicyos deppei*, *Acacia sedillense*, *Sebastiania adenophora*, AND *Lantana camara*[a]

Protein (kDa)	pI	*S. deppei*	*A. sedillense*	*S. adenophora*	*L. camara*
70.0	5.5		E		D
34.0	6.9	D	I	I	
30.0	6.3	D			
29.0	6.2			I	I
26.0	6.7			I	I
25.0	6.8		I		I
23.0	7.0	D		D	
17.0	6.0	E		E	

[a] I = increased; D = decreased; E = repressed.

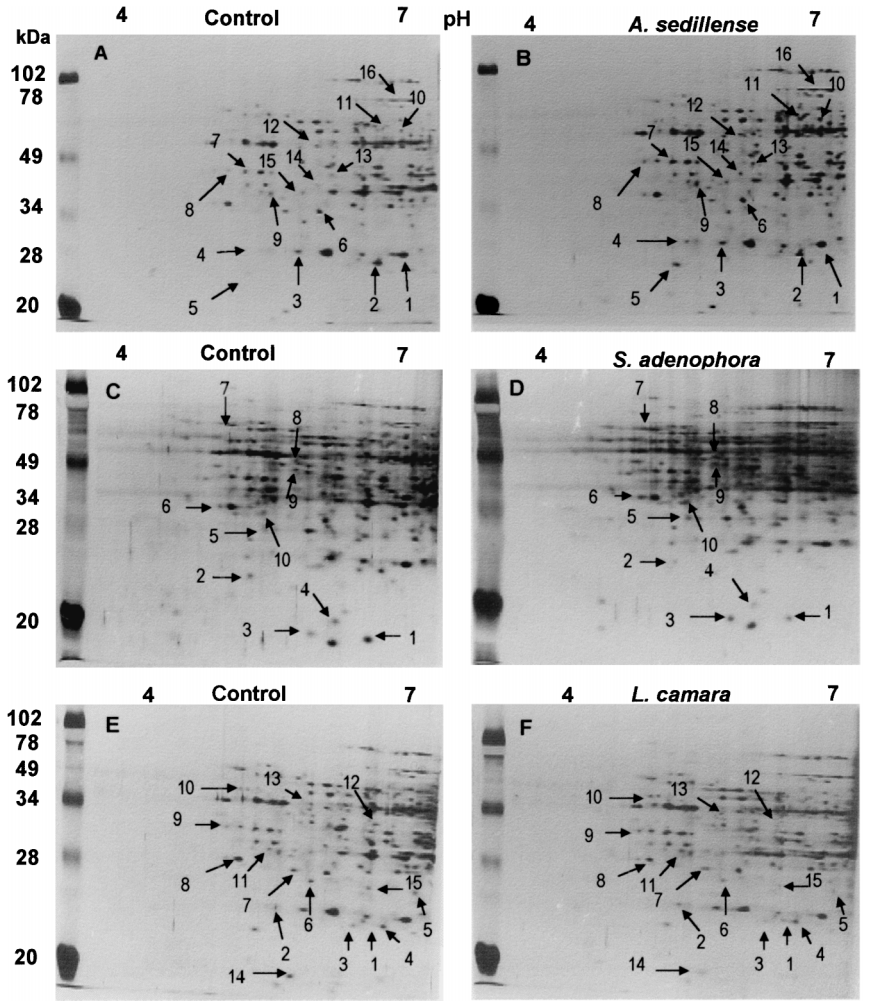


FIG. 3. 2D-PAGE of cytoplasmic root proteins from control bean seedlings (A, C, and E) and those subjected to the aqueous leachates: *A. sedillense* (B), *S. adenophora* (D), and *L. camara* (F). Ten micrograms of the proteins were loaded in each gel. Arrows indicate those proteins that were altered and detected by gel scanning. Gels represent the mean of at least three replicates. Molecular masses in kilodaltons (kDa) are indicated.

DISCUSSION

The metabolic stress response is an ubiquitous defense mechanism activated when cells are confronted with unfavorable environmental conditions. Induction leads to the expression of proteins known as stress proteins, which are thought

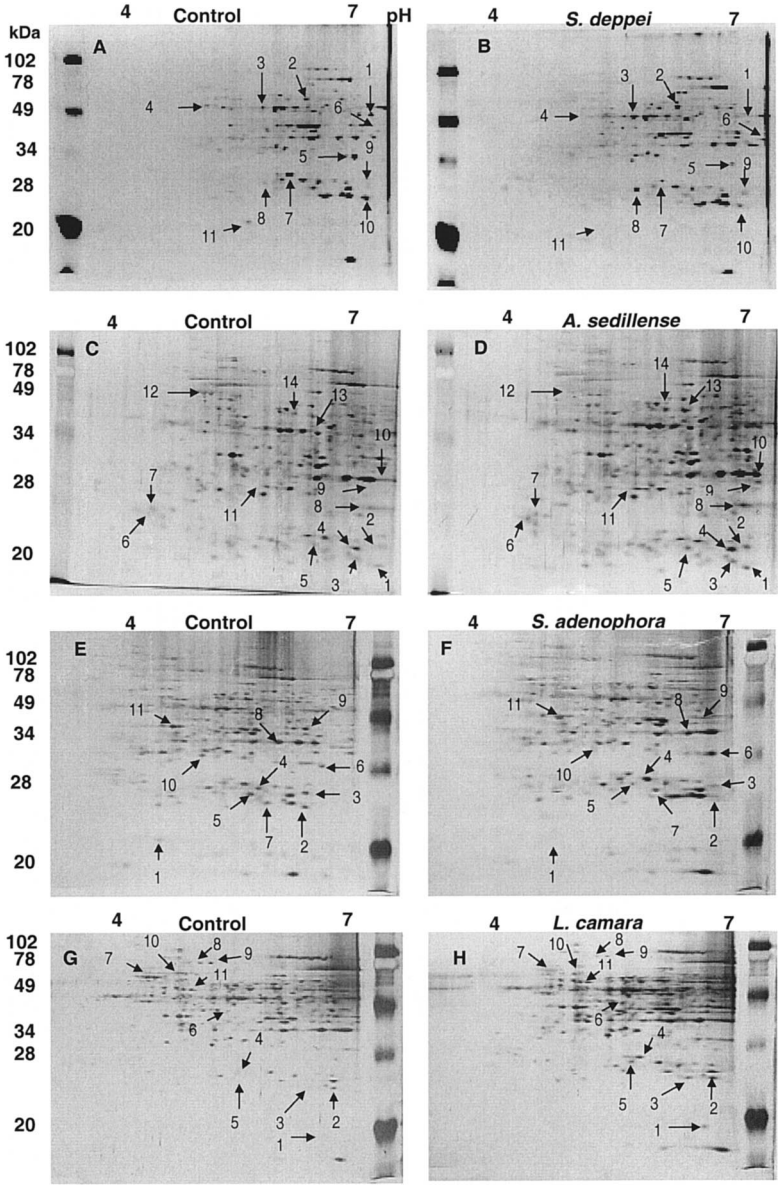


FIG. 4. 2D-PAGE of cytoplasmic root proteins from control tomato seedlings (A, C, E, and G) and those subjected to the aqueous leachates: *S. deppei* (B), *A. sedillense* (D); *S. adenophora* (F); and *L. camara* (H). Ten micrograms of the proteins were loaded in each gel. Arrows indicate those proteins that were altered and detected by gel scanning. Gels represent the mean of at least three replicates. Molecular masses in kilodaltons (kDa) are indicated.

to protect the cell. Stress and signaling inside the cell lead to protein expression changes, the activation of new biochemical pathways, and repression of others that are characteristic of the unstressed state (Bohnert and Sheveleva, 1998). The allelochemicals produced by the plants reported in this study, most of them of unknown chemical nature, may cause stress. Dayan et al. (2000) asserted that determination of the mode of action of allelochemicals is a challenging endeavor due to the multitude of potential molecular targets. However, this seemingly overwhelming task also presents an opportunity to discover new sites of action. In this sense, the present study adds to our understanding.

The effects of the leachates on the crop plants investigated varied in both radicle growth and cytoplasmic protein synthesis. Maize was the most chemical stress tolerant crop plant (inhibited by only *S. deppei*), and tomato the least (inhibited by all allelochemical plants). All the crop plants exhibited changes in the expression of their root cytoplasmic proteins. Overall there was a general increase in protein expression, some reduction, and little induction or repression. *A. sedillense* and *L. camara* leachates affected the largest number of proteins, resulting in a general increase in protein expression. *S. deppei* affected the least and produced a significant change only in tomato. *L. camara* caused a significant inhibition of radicle growth of *C. pepo*, but resulted in the highest decrease of proteins in *P. vulgaris*.

It is possible that the proteins that increased or were induced in response to the treatments are stress proteins that are related to defense mechanisms. They may be formed as a response to allelochemical stress, as are heat shock proteins (HSPs), pathogenic-related proteins (PR), or salinity and dehydration proteins (osmotin, LEA, dehidrins, among others) (Vierling, 1991; Bray, 1993; Van Loon et al., 1994). Proteins whose expression either decreased or were repressed may be those involved in "housekeeping processes," such as cell division or elongation, cell wall formation, or vesicle trafficking. Riccardi et al. (1998) reported a decrease in the expression of certain proteins when maize leaves were subjected to water stress. Sequencing of these peptides showed they were enzymes of the Krebs cycle and glycolysis, such as triose-phospho isomerase, enolase, and NAD-malate-dehydrogenase. Baziramakenga et al. (1997) found that some phenolic acids alter the incorporation of ^{32}P into DNA and RNA and reduce the uptake of [^{32}S] methionine into root soybean seedlings proteins.

S. deppei inhibited radicle growth significantly in maize and beans, but interestingly it did not modify the protein synthesis pattern. In a previous study, we observed that *S. deppei* caused plasma membrane disruption in root tips and plasmolized cells in the peripheral zone of beans and bottle gourd roots (Cruz-Ortega et al., 1998), suggesting that it may be altering membrane processes. It would be interesting to study the effects of this plant on electrolyte leakage and enzyme activities.

This study is one of the first describing changes in protein synthesis patterns caused by allelochemicals from phytotoxic plants. The proteins that were

significantly affected are currently being microsequenced to understand their function. In addition, isolation and identification of the major phytotoxic compounds are underway so that we can test the effects of individual compounds.

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EFFECTS OF WATER DEFICIT STRESS, SHADE, WEED COMPETITION, AND KAOLIN PARTICLE FILM ON SELECTED FOLIAR FREE AMINO ACID ACCUMULATIONS IN COTTON, *Gossypium hirsutum* (L.)

ALLAN T. SHOWLER*

Kika de la Garza Subtropical Agricultural Research Center, USDA-ARS
2413 E. Highway 83, Bldg. 201
Weslaco, Texas 78596

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Abstract—Leaves of cotton plants, *Gossypium hirsutum* L., stressed by water deficit, reduced daylight, and weed competition, or treated with a kaolin wettable powder formulation were analyzed for levels of 17 free amino acids (FAAs) using reversed-phase high-performance liquid chromatography. Water deficit stress resulted in heightened free proline levels (49.9-fold, $P < 0.001$) that were correlated with diffusive resistance (seconds per centimeter). Five other FAAs increased, and the amounts of total free essential (for insect growth and development) amino acids and total FAAs also increased ($P \leq 0.05$). Cotton grown in 50% shade accumulated significantly more free arginine than control plants. In a small-plot weed competition assay, four FAAs increased and three FAAs decreased in association with weed competition, but because free proline levels were not altered and free arginine levels increased, other stresses aside from water deficit, possibly including shading by tall weeds, appear to have caused the changes. In a small-plot kaolin particle film assay, five FAAs were lower in cotton foliage sprayed weekly with kaolin. Because free proline was unaffected and free arginine was lower, it is possible that kaolin's reflectivity heightened light reception. The responses of free proline and arginine to the treatments used in these assays demonstrate that types and degrees of some stresses to cotton can be characterized by accumulations of certain FAAs. The study also demonstrates how some FAA levels can indicate degrees of cotton stress resulting from weed competition and from kaolin particle film application. Porometry and leaf water potential measurements assisted in corroborating some findings of the study.

Key Words—*Gossypium hirsutum*, cotton, free amino acids, water deficit, kaolin, weed competition, plant stress.

*E-mail: ashowler@weslaco.ars.usda.gov

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INTRODUCTION

Moisture deficit stress in plants has been shown to induce changes in plant free amino acid (FAA) accumulations (Bussis and Heineke, 1998). Changes of other stress-inducing parameters have also been associated with altered FAA contents, including soil or water salinity (Martinez et al., 1994), weed competition (Showler et al., 1991), and viral (Showler et al., 1990), bacterial (Meon et al., 1978), fungal (Nemec, 1995), and nematode (Doney et al., 1970) infections. Proline appears to be the FAA that reflects water deficit stress the most consistently by accumulating to significantly higher levels (Gzik, 1996).

Shading significantly decreased cotton leaf photosynthetic rate and nonstructural carbohydrate concentration, increased concentrations of chlorophyll and mineral nutrients, and reduced boll numbers (Zhao and Oosterhuis, 1998, 2000). Intermittent shade was shown to cause stomates to reacclimate within 4 min to changing light conditions to compensate for associated changes in transpiration rates (Bauer et al., 1997). Similar to water deficit, light reduction in cotton could be associated with distinctive FAA patterns.

Kaolin is a white, nonporous, nonswelling, nonabrasive, fine-grained platy aluminosilicate mineral [$Al_4Si_4O_{10}(OH)_8$] that disperses in water and is chemically inert over a wide pH range. Coating-grade kaolin is $>$90% pure and has a high brightness quality of $>$85% (Harben, 1995). Application of kaolin to crops has been shown to reduce damage caused by some plant pathogens and insects (Glenn et al., 1999), including the boll weevil, *Anthonomus grandis* Boheman (Showler, 2001a), an important pest of cotton. Kaolin can act as a physical barrier that makes the host plant visually or tactilely unrecognizable, and insect movement and feeding can also be hindered by the particles; the hydrophobic quality prevents pathogen propagules from contacting the leaf surface (Glenn et al., 1999). In addition to its possibilities for suppression of pest injury to crops, studies have shown how kaolin does not reduce photosynthesis in apple, peach, and pear, with particle levels up to 3000 μg/cm^2 on the leaf surfaces, and canopy temperatures significantly declined because of infrared radiation reflection from the leaf surfaces (Glenn et al., 1999).

This study was conducted to identify FAAs that are influenced by water deficit and shade stresses in cotton, *Gossypium hirsutum* L., and to interpret FAA levels observed in weed competition stressed and in kaolin-coated plants using FAA measurements from the water deficit and shade assays.

METHODS AND MATERIALS

Water Deficit Assay, Part 1. Eighty 7.6-liter pots with Sunshine mix No. 1 nursery potting soil ($\sim$75% sphagnum peat moss, perlite, dolomitic limestone, and gypsum; Sungro Horticulture, Bellevue, Washington) were each planted on May 3, 2000, with five cotton (var. C-208) seeds. Seedlings were thinned to three

per pot, and 200 ml of Peters Professional (Scotts-Sierra Horticultural Products Company, Marysville, Ohio) water-soluble general purpose 20-20-20 N-P-K fertilizer at 15.8 g/liter of water was applied to each pot at the two true leaf stage in this and all other greenhouse assays in the study. All pots were irrigated with 1 liter of water every week until the cotton plants reached the four true leaf stage. Then, four groups of 20 pots/group were maintained on separate irrigation regimes: 4000 ml water/week (saturation), 1500 ml/week, 1000 ml/week, and 750 ml/week, each delivered at one time per week. All the pots were placed in 4-cm-deep plastic dishes to retain any water, especially in the saturated regime, that might have run through the soil and out the holes in the bottom of the pots. After one week, irrometers for monitoring soil moisture in the pots were discontinued because the periodically dry soil in most pots prevented an adequate soil moisture–irrometer interface. The cotton plants were grown to the 7- to 10-mm-square diameter stage, then on June 19, 2000, an LI 1600 Steady State Porometer (Li-cor, Inc., 1989) was used to measure diffusive resistance and transpiration rates from upper (top four fully expanded) leaves on 17 of the plants in each water regime (changing cloud cover prevented further measurements). Twenty-five upper leaves grown in 2001 under approximately the same conditions were excised and their water potentials measured with a Model 610 (PMS Instrument Co., Corvallis, Oregon) pressure bomb on June 21, 2001. On the same day, 1 g fresh wt from the distal halves of upper cotton leaves (0.5 g from each of four leaves) from each pot ($n = 20$) was excised and homogenized with 10 ml 0.1 N HCl using a Virtishear homogenizer (Virtis, Gardiner, New York). At least 5 g homogenate from each sample was placed in separate 10-ml tubes and centrifuged at 10,000 rpm for 30 min. Samples were stored at -80°C.

One milliliter of supernatant from each sample was filtered through a 0.5-μl filter fitted to a 5-ml plastic syringe. Samples were placed in the autosampler of an Agilent 1100 Series (Agilent Technologies, Atlanta, Georgia) reversed-phase high-performance liquid chromatograph (HPLC) with a binary pump delivering solvent A [1.36 g sodium acetate trihydrate + 500 ml purified HPLC grade water + 150 μl triethylamine (TEA) + sufficient acetic acid to bring the pH to 7.2 ± 0.05] and solvent B [1.36 g sodium acetate trihydrate + 100 ml purified HPLC grade water (acetic acid added to this mixture to bring the pH to 7.2 ± 0.05) + 200 ml acetonitrile + 200 ml methanol] at 0.3 and 0.45 ml/min on a concave gradient through an Eclipse XDB-C8 4.6 mm 10×15 cm C_{18} fluorescence column (Agilent Technologies). Absorbances at 262 and 338 nm were monitored on a variable wavelength detector for 40 min per sample. The autosampler measured and mixed 6 μl sodium borate buffer (0.4 N, pH 10.2 in water), 1 μl 9-fluorenylmethylchloroformate (FMOC), and 1 μl ophthalaldehyde (OPA) derivatizing agents, and 2 μl of sample, then injected 2 μl for chromatographic separation of FAAs. Identification and quantification of derivatized FAAs were achieved by calibrating with a standard mixture of amino acids. Peak integration accuracy was enhanced by manual establishment of peak baselines using Agilent software.

Water Deficit Assay, Part 2. In the greenhouse, C-208 cotton was grown in 7.6-liter pots as described in part 1. Upon reaching the four true leaf stage, cotton plants received 1000, 750, 500, and 250 ml water/week ($n = 20$), and 20 pots of cotton were irrigated with 1000 ml water biweekly. Each dose of water was delivered in one irrigation weekly or biweekly. Twenty other cotton pots were provided 1000 ml water/week, but alfalfa, (*Medicago sativa* L.) seedlings were also grown at a density of 3/cm^2 in order to observe effects of competition for limited water. At the 7- to 10-mm-diam. square stage, 1 g of leaf material was excised, homogenized, stored, and analyzed for FAA content as described in part 1. Diffusive resistance and transpiration rate measurements were taken from the upper leaves of one plant in each pot by using a porometer. Water potential measurements were not taken because of broken instrumentation.

Shade Assay. In a greenhouse (to better control potential effects of other environmental parameters that could also affect FAA levels), October 11–November 28, 2000, 20 pots of C-208 cotton plants at the four leaf stage were covered with 50% shade cloth suspended 3.5 m overhead and along all four sides. Twenty control pots were not shaded. All received 1500 ml water each week beginning at the four-leaf stage. Water potential of one upper leaf from 25 plants grown in 2001 under approximately the same conditions was measured with the pressure bomb on June 15, 2001. Plant heights were recorded from 25 plants in each treatment on November 27, 2000, and FAA sample collection and analyses were as described in the water deficit assay, part 1. Porometry measurements were not taken because full sunlight is recommended (Li-cor, 1989).

Weed Competition Assay. A field at the USDA-ARS Kika de la Garza Subtropical Agricultural Research Center in Hidalgo County, Texas, was divided into twelve 0.028-ha plots and planted to Deltapine 436 RR cotton (this variety was planted because glyphosate was used to eliminate weeds on the rows) on March 6, 2000. In a randomized design, six of the 12 plots received a topical application of pendamethalin at 924 g-active ingredient (ai)/ha at planting; a rolling cultivator, glyphosate spot treatments, and hand-roguing were used thereafter to maintain weed-free plots. Weeds were permitted to grow freely in six weedy plots. Weed dry biomass was determined on April 18 and June 1 with two 0.5-m^2 quadrats per plot. The weeds were clipped at the soil surface, separated by species, oven dried for 48 hr at 60°C, and then weighed.

On May 15, 2000, when the cotton plants were squaring, the porometer was used to measure diffusive resistance and transpiration rates on five upper leaves in each of the 12 plots. On the same day, fifteen 1-g samples of leaf material were excised from each treatment and placed on ice until arrival at the lab within 5 min. The samples were homogenized, stored, and analyzed for FAA accumulations as described in the water deficit assay, part 1. Heights of five cotton plants in each plot were recorded (from soil level to uppermost leaf) on May 15, 2000. On July 12, two 4-m sections of row in each plot were hand-harvested, ginned, and the lint

weighed to determine yield. Water potentials of two upper leaves grown in 2001 under approximately the same conditions in each of the six treatment replicates were measured with the pressure bomb on June 26, 2001.

Kaolin Particle Film Assay. A field at the USDA-ARS Kika de la Garza Subtropical Agricultural Research Center, Hidalgo County, Texas, was arranged in a randomized design with sixteen .012-ha plots, and planted to C-208 cotton on March 6, 2000; pendimethalin (924 g ai/ha) was applied for weed control on the same day. Weeds were controlled thereafter using a rolling cultivator and hand-roguing, and irrigation occurred once at preplanting. Eight of the plots were not treated with kaolin (check), and eight plots were given a double application of 22.7 kg Surround WP (Engelhard, Iselin, New Jersey) per 378 liters of water from a tractor-mounted spray boom weekly from April 11 until mid-June 2000.

Kaolin particle deposition on the cotton leaves was determined by excising 20 leaves at random from each treatment. Kaolin particles were washed with methanol into preweighed plastic dishes (a 6-mm flat ox-hair paint brush was used to dislodge particles adhering to leaf surfaces), the methanol was evaporated off, and the dried particles plus dish were weighed. The upper surface area of each leaf was measured with a model 3100 Area Meter (Li-cor, Lincoln, Nebraska). The mass of kaolin collected was divided by two times the leaf area on one side to give the mass of kaolin deposited per square centimeter leaf surface area. Porometry measurements were not taken because the presence of particles on the leaf surfaces can reduce accuracy (Idso et al., 1987). Water potentials of two upper leaves from each of the eight treatment replicates grown in 2001 under approximately the same set of conditions were measured with the pressure bomb on June 26, 2001.

On May 19, 2000, when the cotton plants were blooming, two sets of three upper leaves per plot were collected, placed on ice, and taken to the lab within 5 min. Each three-leaf sample was used to obtain 1.0 g of leaf material that was homogenized, stored, and analyzed for FAA contents in the same manner as described for the water deficit assay, part 1. Heights of 200 cotton plants were measured from soil level to uppermost leaf, on May 15, 2000. On July 7, two 4-m sections of row in each plot were hand harvested, ginned, and the lint weighed.

Statistical differences among the treatments in these experiments were detected by using the two-sample *t* test or ANOVA (randomized design) and Tukey's HSD for mean separation (Analytical Software, 1998).

RESULTS

Water Deficit Assay, Part 1. Seventeen FAAs could be detected by using a FAA standard of alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine,

TABLE 1. FREE AMINO ACID[a] (FAA) CONCENTRATIONS (± SE) IN COTTON, *Gossypium hirsutum* L. (VAR. C-208), UPPER LEAVES RESULTING FROM CONTINUOUSLY WATER-SATURATED POTTING SOIL, AND 1500, 1000, AND 750 ml WATER PER WEEK

FAA	Concentration[b] (pmol/μl extract)			
	Saturated	1500 ml/week	1000 ml/week	750 ml/week
Alanine	857.2 ± 60.0 b	1,299.2 ± 43.2 a	1,264.2 ± 54.6 a	1,423.2 ± 58.1 a
Arginine	862.9 ± 74.9 b	2,347.2 ± 413.4 a	2,702.8 ± 473.8 a	3,462.8 ± 335.2 a
Aspartic acid	2,097.6 ± 171.9	1,694.4 ± 142.5	2,110.5 ± 169.6	2,032.9 ± 147.3
Glutamic acid	1,896.0 ± 130.0	2,079.6 ± 84.9	1,995.4 ± 156.2	2,082.5 ± 103.3
Glycine	46.6 ± 4.6 b	89.9 ± 17.0 a	61.1 ± 7.6 ab	70.9 ± 7.4 ab
Histidine	28.7 ± 10.2 c	117.4 ± 30.5 bc	187.2 ± 46.6 b	330.7 ± 30.3 a
Isoleucine	675.8 ± 29.3 c	1,732.2 ± 9.1 a	1,106.9 ± 107.0 b	1,080.5 ± 84.7 b
Phenylalanine	137.7 ± 19.3 c	203.9 ± 30.8 bc	296.0 ± 45.1 ab	421.8 ± 41.2 a
Proline	22.4 ± 1.9 c	186.7 ± 43.2 bc	589.4 ± 127.3 b	1,116.1 ± 174.5 a
Serine	402.9 ± 29.0 b	628.6 ± 31.4 a	438.6 ± 45.2 b	429.7 ± 28.6 b
Threonine	517.5 ± 45.1	568.3 ± 25.5	643.6 ± 72.7	648.7 ± 33.3
Valine	118.4 ± 6.0 c	303.7 ± 39.1 b	311.2 ± 53.0 b	452.7 ± 34.2 a
Total essential FAAs	2,362.4 ± 111.9 b	5,259.8 ± 490.3 a	5,264.2 ± 646.8 a	6,397.2 ± 405.2 a
Total FAAs	7,499.6 ± 449.7 b	11,775.0 ± 879.7 a	11,723.0 ± 958.3 a	13,552.2 ± 633.4 a

[a] Free cysteine/cystine, leucine, lysine, methionine, and tyrosine were detectable but not present in the cotton extracts analyzed.
[b] Means followed by different letters in the same row are significantly different ($P \leq 0.05$), $n = 20$.

threonine, tyrosine, and valine. Of the 17 detectable FAAs, 12 were found in cotton leaf extract: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, phenylalanine, proline serine, threonine, and valine (Table 1), although histidine and phenylalanine appeared to be absent in some samples. Of the 10 amino acids essential for insect growth and development (Vanderzant, 1958), six were detected in cotton: arginine, histidine, isoleucine, phenylalanine, threonine, and valine.

The four moisture regimes were associated with significant ($P \leq 0.05$) differences in all detectable FAA concentrations, except glutamic acid. Arginine ($F = 26.08, df = 5$), aspartic acid ($F = 3.64, df = 5$), glycine ($F = 3.47, df = 5$), and threonine ($F = 4.94$, $df = 5$) levels were lower ($P \leq 0.05$) in the plants irrigated with 4000 ml/week than the plants irrigated with 1500, 1000, and 750 ml/week.

Six (50%) of the detectable FAAs in cotton foliage, arginine, histidine, phenylalanine, proline, threonine, and valine, increased with higher water deficit stress. In the case of threonine, the difference between saturated and 750 ml/week watering regimes was relatively low and the difference was marginal (1.2-fold, $F = 1.76$, $df = 3$, $P = 0.163$), but the five other FAA accumulations increased 3.1 times (phenylalanine, $F = 12.0$, $df = 3$, $P \leq 0.05$) to 49.9 times (proline, $F = 19.56$,

$df = 3$, $P \leq 0.05$). Levels of free isoleucine and serine were highest in the 1500 ml/week regime, and lower with each drier regime, but lowest in the water saturated regime.

Total free essential amino acids in the saturated and 1500, 1000, and 750 ml/week regimes comprised 31.5%, 44.7%, 44.9%, and 47.2% of the total FAA accumulations, respectively. Free arginine comprised 36.5%, 44.6%, 51.3%, and 54.1% of the detectable free essential amino acids, respectively.

Diffusive resistance in the 750 ml/week regime cotton was 31 times that of cotton grown in water saturated soil ($F = 14.60$, $df = 3$, $P \leq 0.05$) (Table 2). Similarly, 1000 ml/week and 1500 ml/week regime cotton were found to have 24.2 times

TABLE 2. DIFFUSIVE RESISTANCE, TRANSPIRATION RATES, AND WATER POTENTIALS OF COTTON LEAVES ON PLANTS IN WATER DEFICIT, SHADE, WEED COMPETITION, OR KAOLIN PARTICLE FILM ASSAYS[a]

Assay and treatment	Diffusive resistance (sec/cm)	Transpiration rate (μg/cm^2/sec)	Water potential (barr)[b]
Water deficit, part 1[c]			
Saturated	0.4 ± 0.04 c	24.5 ± 1.1 a	10.2 ± 0.2 b
1,500 ml/week	7.2 ± 1.4 b	4.8 ± 0.8 b	11.3 ± 0.6 b
1,000 ml/week	10.6 ± 2.0 ab	5.2 ± 1.1 b	30.4 ± 0.8 a
750 ml/week	13.5 ± 1.5 a	2.6 ± 0.3 b	32.5 ± 0.8 a
Water deficit, part 2[d]			
1,000 ml/week	9.7 ± 0.5 d	6.4 ± 0.5 a	
750 ml/week	12.0 ± 0.6 cd	4.9 ± 0.2 b	
500 ml/week	13.4 ± 0.3 c	4.0 ± 0.3 bc	
250 ml/week	18.2 ± 0.9 b	2.8 ± 0.2 c	
1,000 ml biweekly	22.4 ± 0.8 a	1.1 ± 0.2 d	
1,000 ml/week + alfalfa	11.9 ± 0.7 cd	5.0 ± 0.5 b	
Shade			
50% shade			12.2 ± 0.4
Full sun			11.3 ± 0.6
Weed competition[e]			
Weedy	2.2 ± 0.6	4.8 ± 0.4 b	11.3 ± 0.6
Weed-free	0.8 ± 0.1	7.5 ± 0.6 a	11.8 ± 1.0
Kaolin particle film			
Kaolin			8.2 ± 0.3
No kaolin			8.5 ± 0.3

[a] Values are means ± SE. Means compared only within each assay; means followed by different letters are significantly different ($P \leq 0.05$). Leaf water potential measurements were taken on plants grown in 2001 under approximately the same sets of conditions. —, no data taken.
[b] $n = 25$.
[c] $n = 17$.
[d] $n = 6$.
[e] Water deficit and shade assays, $n = 25$; weed competition assay, $n = 6$; kaolin particle film assay, $n = 8$.

($P \leq 0.05$) and 16.6 times ($P \leq 0.05$) higher diffusive resistances as compared to water saturated cotton. Transpiration rate (micrograms of water per square centimeter per second) in the water saturated cotton, inversely influenced by diffusive resistance, was 5.1 ($F = 137.58$, $df = 3$, $P \leq 0.05$), 4.7 ($P \leq 0.05$), and 9.4 times ($P \leq 0.05$) that of the 1500, 1000, and 750 ml/week cotton, respectively. Water potentials in the 750 and 1000 ml/week regime leaves were $\geq$63% ($F = 333.57$, $df = 3$, $P \leq 0.05$) higher than in the 1500 ml/week and saturated regimes (Table 2).

Water Deficit Assay, Part 2. In relation to the 1000 ml/week regime, the highest levels ($P \leq 0.05$) of five FAAs occurred in the biweekly regime: arginine (6.4-fold, $F = 26.08$, $df = 5$), glycine (3.7-fold, $F = 3.47$, $df = 5$), proline (16.0-fold, $F = 26.64$, $df = 5$), serine (3.1-fold, $F = 5.93$, $df = 5$), and valine (4.3-fold, $F = 24.17$, $df = 5$) (Table 3). Free essential amino acids and total FAA accumulations in the biweekly regime were also significantly ($P \leq 0.05$) higher (2.4 times, $F = 25.35$, $df = 5$; 1.6 times, $F = 17.04$, $df = 5$, respectively) than in the 1000 ml/week regime. Among the FAA responses to the 1000, 750, 500, and 250 ml/week regimes, six followed one of two nonlinear patterns. The first, reflected by free arginine, histidine, proline, and valine, increased from the 1000 to 750 ml/week regimes, stabilized from 750 to 500 ml/week, and increased from 500 to 250 ml/week. The second pattern, reflected by aspartic acid, glutamic acid, and threonine, decreased from 1000 ml/week to the lowest levels at 750 ml/week, and increased from 750 to 250 ml/week. Total free essential amino acids generally conformed to the first pattern and total FAAs resembled the second pattern.

From 1000 to 250 ml/week, levels of six FAAs increased ($P \leq 0.05$): arginine (5.8-fold, $F = 26.08$, $df = 5$), glycine (2.8-fold, $F = 3.47$, $df = 5$), histidine (6.2-fold, $F = 22.42$, $df = 5$), phenylalanine (3.5-fold, $F = 25.19$, $df = 5$), proline (17.2-fold, $F = 26.64$, $df = 5$), and valine (3.6-fold, $F = 24.17$, $df = 5$). Total free essential amino acids and total FAAs increased ($P \leq 0.05$) by 3.4-fold ($F = 25.35$, $df = 5$) and 1.5-fold ($F = 17.04$, $df = 5$), respectively.

The 1000 ml/week + alfalfa seedlings resulted in concentrations of aspartic acid, glutamic acid, histidine, proline, serine, threonine, valine, total free essential amino acids, and total FAAs that were most similar to 750 or 500 ml/week levels (Table 3). Concentrations of glycine and phenylalanine in the 1000 ml/week + alfalfa regime were most similar to accumulations in 1000 ml/week cotton.

Percentage of total free essential amino acids to total FAAs was lowest in the 1000 ml/week regime (10.6%), highest in the 1000 ml biweekly regime (24.9%), and second highest in the 250 ml/week regime (23.9%). The percentage of total free essential amino acids in the 1000 ml/week + alfalfa regime (17.6%) was most similar to that of the 750 ml/week regime (17.0%). Free arginine was the predominant free essential amino acid, except in the 1000 ml/week foliage where free isoleucine levels were 1.2 times higher ($P \geq 0.05$) than levels of free arginine.

Diffusive resistance in the 1000-ml biweekly regime cotton was 2.3-fold higher ($F = 50.74$, $df = 5$, $P \leq 0.05$) than the 1000 ml/week regime cotton

TABLE 3. FAA CONCENTRATIONS IN COTTON, *Gossypium hirsutum* L. (VAR. C-208), UPPER LEAVES RESULTING FROM 1000, 750, 500, AND 250 ml WATER WEEKLY, 1000 ml WATER BIWEEKLY, AND 1000 ml WATER WEEKLY WITH THREE ALFALFA SEEDLINGS PER SQUARE CENTIMETER

FAA	Concentration (pmoles/μl extract)[a]					
	1000 ml/week	750 ml/week	500 ml/week	250 ml/week	1000 ml biweekly	1000 ml/week + alfalfa
Alanine	1,202.0 ± 68.4 a	917.7 ± 32.7 b	1,315.2 ± 187.0 a	1,249.5 ± 46.8 a	1,164.7 ± 115.1 ab	1,080.6 ± 39.6 ab
Arginine	386.0 ± 35.0 c	1,116.0 ± 50.4 b	929.4 ± 99.2 bc	2,232.8 ± 149.7 a	2,480.1 ± 333.5 a	982.1 ± 84.0 bc
Aspartic acid	6,346.4 ± 303.1 a	5,024.2 ± 308.4 b	5,196.5 ± 198.9 ab	5,907.8 ± 376.3 ab	6,400.9 ± 353.7 a	5,446.4 ± 275.5 ab
Glutamic acid	6,612.8 ± 237.5 ab	5,631.5 ± 254.2 bc	6,201.4 ± 186.3 ab	7,232.2 ± 487.8 a	7,221.4 ± 627.2 a	4,546.4 ± 169.6 c
Glycine	41.4 ± 7.2 b	41.6 ± 7.0 b	96.8 ± 14.7 ab	1,16.8 ± 39.8 ab	151.7 ± 46.9 a	33.1 ± 4.6 b
Histidine	150.1 ± 26.5 b	301.3 ± 31.7 b	265.6 ± 43.4 b	924.3 ± 95.2 a	824.8 ± 118.4 a	335.8 ± 36.0 b
Isoleucine	446.9 ± 48.6 b	497.4 ± 40.4 b	377.3 ± 36.3 b	626.9 ± 49.2 ab	811.6 ± 137.2 a	589.3 ± 29.7 ab
Phenylalanine	244.8 ± 17.9 b	226.5 ± 25.8 b	193.8 ± 31.5 b	850.1 ± 103.7 a	1,083.7 ± 150.0 a	277.9 ± 13.4 b
Proline	209.4 ± 19.9 b	1,051.5 ± 165.8 b	1,044.0 ± 303.8 b	3,588.2 ± 377.4 a	3,359.8 ± 427.6 a	608.8 ± 180.0 b
Serine	340.9 ± 41.3 b	651.3 ± 37.6 b	618.2 ± 54.3 b	644.2 ± 45.2 b	1,048.2 ± 205.8 a	613.6 ± 38.3 b
Threonine	348.2 ± 41.1 a	275.8 ± 19.7 ab	306.9 ± 29.5 a	346.5 ± 16.3 a	292.2 ± 27.2 ab	186.7 ± 18.6 b
Valine	217.8 ± 29.1 b	258.9 ± 22.1 b	257.9 ± 33.2 b	793.7 ± 84.4 a	931.8 ± 122.9 a	262.1 ± 28.3 b
Total essential FAAs	1,750.3 ± 124.4 b	2,690.0 ± 137.6 b	2,330.9 ± 203.9 b	5,873.2 ± 433.2 a	6,424.1 ± 810.1 a	2,633.3 ± 154.6 b
Total FAAs	16,505 ± 588 b	15,854 ± 544 b	16,803 ± 731 b	24,592 ± 1565 a	25,821 ± 2,070 a	14,952 ± 440 b

[a] Values are means ± SE. Means followed by different letters in the same row are significantly different ($P \leq 0.05$), $n = 20$.

(Table 2). Reductions of water in the weekly irrigation regimes reflected increases in diffusive resistance, which at 250 ml/week was 1.9 times higher ($F = 50.74$, $df = 5$, $P \leq 0.05$) than that of cotton grown at 1000 ml/week. The Pearson correlation value for diffusive resistances and free proline accumulations was 0.98 ($P = 0.024$). Cotton irrigated in the 1000 ml/week regime had a transpiration rate 5.9 times ($F = 33.49$, $df = 5$, $P \leq 0.05$) that of cotton in the 1000-ml biweekly regime, and 2.3 times ($P \leq 0.05$) that of cotton in the 250 ml/week regime. Diffusive resistance and transpiration rates of cotton in the 1000 ml/week + alfalfa regime were approximately the same as those in the 750 ml/week cotton.

Shade Assay. Cotton plants grown under 50% shade were taller (45%, $t = 6.51$, $df = 48$, $P < 0.001$) than unshaded plants (Table 4). In conditions of full sunlight, accumulations of free aspartic acid and arginine were 1.2 times ($t = 2.40$, $df = 38$, $P = 0.021$) and 0.5 times ($t = 8.96$, $df = 38$, $P < 0.001$), respectively, the amounts found in the shaded leaves (Table 4). There were no differences among any of the other detectable FAA levels. Leaf water potentials were not higher in the shaded cotton (Table 2).

Weed Competition Assay. Total weed biomass was comprised of pigweed, *Amaranthus palmeri* S. Wats., (36.1%) (average percentages from April 18 and June 1); *Brachiaria texana* (Buckl.) Blake, (55.7%); ragweed, *Ambrosia*

TABLE 4. FREE AMINO ACID CONCENTRATIONS IN COTTON, *Gossypium hirsutum* L. (VAR. C-208), UPPER LEAVES RESULTING FROM 50% DAYLIGHT REDUCTION AND NO DAYLIGHT REDUCTION

	Concentration (pmoles/μliter extract)[a]	
FAA	50% shade	No shade
Alanine	1,089.0 ± 71.0	930.6 ± 81.6
Arginine	256.2 ± 11.8 a	125.3 ± 8.6 b
Aspartic acid	3,484.1 ± 224.4 b	4,268.7 ± 238.0 a
Glutamic acid	7,071.2 ± 345.9	7,186.8 ± 337.4
Glycine	22.5 ± 1.7	23.0 ± 3.0
Histidine	73.3 ± 6.0	91.4 ± 18.1
Isoleucine	170.8 ± 32.9	225.5 ± 58.5
Phenylalanine	198.9 ± 18.8	202.5 ± 27.7
Proline	18.9 ± 2.4	23.2 ± 3.4
Serine	171.8 ± 29.8	175.1 ± 11.6
Threonine	111.5 ± 10.4	109.0 ± 11.1
Valine	115.7 ± 14.8	92.8 ± 10.3
Total essential FAAs	921.6 ± 51.4	846.5 ± 79.1
Total FAAs	12,773 ± 581.5	13,450 ± 634.5

[a] Values are means ± SE. Means followed by different letters in the same row are significantly different ($P \leq 0.05$), $n = 20$.

TABLE 5. FREE AMINO ACID[a] (FAA) CONCENTRATIONS IN COTTON, *Gossypium hirsutum* L. (VAR. DELTAPINE 436 RR), UPPER LEAVES RESULTING FROM WEED COMPETITION

FAA	Concentration (pmol/μl extract)[b]	
	Weedy	Weed-free
Alanine	619.2 ± 32.5	646.15 ± 37.7
Arginine	4,597.4 ± 681.1 a	371.8 ± 43.2 b
Aspartic acid	2,203.2 ± 65.3 b	2,640.2 ± 88.6 a
Glutamic acid	1,712.0 ± 41.4 b	2,039.8 ± 88.0 a
Glycine	52.8 ± 11.3	38.0 ± 2.6
Histidine	59.3 ± 11.3 a	8.2 ± 4.5 b
Isoleucine	238.0 ± 39.4 a	136.1 ± 10.1 b
Phenylalanine	197.6 ± 20.0 a	90.7 ± 15.0 b
Proline	44.9 ± 8.7	45.7 ± 2.2
Serine	227.1 ± 12.5	255.2 ± 13.5
Threonine	196.6 ± 10.6 b	240.1 ± 8.0 a
Valine	121.1 ± 10.1	132.9 ± 6.8
Total essential FAAs	5,407.9 ± 705.7 a	979.7 ± 44.6 b
Total FAAs	10,263.5 ± 697.7 a	6,667.3 ± 167.0 b

[a] Free cysteine/cystine, leucine, lysine, methionine, and tyrosine were detectable but not present in the cotton extracts analyzed.
[b] Values are means ± SE. Means followed by different letters in the same row are significantly different ($P \leq 0.05$), $n = 15$.

artemisiifolia L., (6.8%); ground cherry, *Physalis heterophylla* Nees, (1.1%); and spurge, *Euphorbia* sp., (0.4%). On May 15, when cotton leaves were excised for FAA analysis, weed coverage of soil surface area was 85%. Weed dry biomass was 22.4 ± 3.3 g/0.5 m^2 and 111.7 ± 19.8 g/0.5 m^2 on April 18 and June 11, respectively, so the dry biomass of weeds on May 15, assuming linear increase over time, would be ≈68 g/0.5 m^2.

Cotton grown in the weedy habitats, as compared to weed-free cotton, had higher levels (1.4- to 12.4-fold) of five FAAs: arginine, glycine, histidine, phenylalanine, and isoleucine (Table 5). Of those, four were significantly different: arginine (12.4-fold, $t = 6.19$, $df = 28$, $P < 0.001$), histidine (7.3-fold, $t = 4.19$, $df = 28$, $P < 0.001$), isoleucine (1.8-fold, $t = 2.50$, $df = 28$, $P = 0.018$), and phenylalanine (2.2-fold, $t = 4.29$, $df = 28$, $P < 0.001$). Total free essential amino acids and total FAAs were more abundant in the weedy cotton foliage (5.5 times, $t = 6.26$, $df = 28$, $P < 0.001$; 1.5 times, $t = 5.01$, $df = 28$, $P < 0.001$). The predominant FAAs in the weedy cotton were arginine (44.8%), aspartic acid (21.5%), and glutamic acid (16.7%). The four free essential amino acids that were more abundant in weedy cotton foliage comprised 52.7% of the total FAAs accumulated.

TABLE 6. COTTON HEIGHTS AND LINT YIELDS FROM WEED COMPETITION AND KAOLIN PARTICLE FILM ASSAYS[a]

Assay	Treatment	Height (cm)[b]	Yield lint (kg/ha)[c]
Weed Competition	Weedy	27.7 ± 1.1 b	48.8 ± 24.8 b
	Weed-free	38.0 ± 0.9 a	460.7 ± 32.4 a
Kaolin	Check	52.6 ± 0.9	515.3 ± 25.0
	Kaolin	50.6 ± 0.6	520.5 ± 18.4

[a] Values are means $\pm$ SE. Means followed by different letter within each assay are significantly different ($P = 0.05$).
[b] Weed competition assay: $n = 30$, sampling date May 15, 2000; kaolin particle film assay: $n = 200$, sampling date May 18, 2000.
[c] Hand harvested and manually ginned. Weed competition assay, $n = 12$; kaolin particle film assay, $n = 16$.

In cotton grown in weed-free habitats, three FAAs were 1.2-fold higher than in weedy habitats: aspartic acid ($t = 4.38$, $df = 28$, $P = 0.001$), glutamic acid ($t = 3.37$, $df = 28$, $P = 0.002$), and threonine ($t = 3.28$, $df = 28$, $P = 0.003$), and the most abundant FAAs in weed-free cotton foliage were aspartic acid (39.6%), glutamic acid (30.6%), and alanine (9.7%). The combined total of free essential amino acids in weed-free cotton foliage was 14.7% of the total FAA content.

The diffusive resistance of leaves in the weedy plots was 2.8 times ($t = 2.20$, $df = 10$, $P = 0.052$) that of the weed-free habitat cotton foliage. Weed-free cotton transpired 1.6 times faster than weedy cotton ($t = 3.83$, $df = 10$, $P = 0.003$). Water potentials were not different among treatments (Table 2).

Cotton in the weed-free plots grew 1.4 times higher ($t = 7.24$, $df = 58$, $P < 0.001$) than weedy cotton by mid-May (Table 6). Lint yield was 9.4 times higher in weed-free plots than in weedy plots ($t = 10.10$, $df = 10$, $P < 0.001$).

Kaolin Particle Film Assay. Four hours after application, kaolin density ranged from 189.9 to 522.8 μg/cm^2, with a mean of 363.0 ± 18.7 μg/cm^2 ($n = 20$). Particle density after one week ranged from 135.5 to 470.1 μg kaolin/cm^2 with a mean of 319.9 ± 20.8 μg/cm^2 ($n = 20$).

Cotton leaves from control and kaolin-treated plots contained the same variety of detectable FAAs (Table 7) as the other assays. The application of kaolin was associated with significant reductions in free alanine (27.0%, $t = 2.88$, $df = 30$, $P = 0.007$), arginine (67.2%, $t = 3.17$, $df = 30$, $P = 0.004$), isoleucine (62.4%, $t = 2.74$, $df = 30$, $P = 0.010$), phenylalanine (36.0%, $t = 3.40$, $df = 30$, $P = 0.002$), threonine (61.0%, $t = 3.21$, $df = 30$, $P = 0.003$), total free essential amino acids (51.4%, $t = 3.22$, $df = 30$, $P = 0.003$), and total FAAs (15.2%, $t = 3.41$, $df = 30$, $P = 0.002$).

The percentage of detectable free essential amino acids declined from 19.8% in the check to 11.4% in the kaolin-treated leaves. Cotton growing in the check plots

TABLE 7. FREE AMINO ACID[a] (FAA) CONCENTRATIONS IN COTTON, *Gossypium hirsutum* L. (VAR. C-208), UPPER LEAVES RESULTING FROM NO TREATMENT (CHECK) AND WEEKLY APPLICATION OF KAOLIN PARTICLE FILM

FAA	Concentration (pmol/μl extract)[b]	
	Check	Kaolin
Alanine	837.4 ± 51.0 a	679.8 ± 19.6 b
Arginine	459.2 ± 97.0 a	150.8 ± 6.4 b
Aspartic acid	2,487.9 ± 116.4	2,355.2 ± 64.0
Glutamic acid	2,637.7 ± 127.1	2,613.8 ± 26.4
Glycine	39.3 ± 2.5	35.6 ± 2.2
Histidine	46.9 ± 6.3	58.8 ± 4.3
Isoleucine	445.1 ± 100.8 a	167.3 ± 9.8 b
Phenylalanine	223.5 ± 22.1 a	142.9 ± 8.5 b
Proline	50.5 ± 7.1	54.8 ± 3.5
Serine	398.0 ± 59.5	284.8 ± 11.0
Threonine	286.5 ± 45.6 a	139.6 ± 4.0 b
Valine	136.1 ± 11.0	136.3 ± 9.1
Total essential FAAs	1,597.3 ± 253.5 a	7,76.9 ± 22.3 b
Total FAAs	8,048.2 ± 348.5 a	6,828.3 ± 82.1 b

[a] Free cysteine/cystine, leucine, lysine, methionine, and tyrosine were detectable but were not present in the cotton extracts analyzed.
[b] Values are means ± SE. Means followed by different letters in the same row are significantly different ($P \leq 0.05$), $n = 16$.

did not differ in height or lint yield per hectare from kaolin-treated cotton (Table 6). Differences in diffuse resistances, transpiration rates, and water potentials were not detected between the two treatments (Table 2).

Overall, each of the 12 detectable FAA accumulations in cotton foliage was significantly altered as a result of one or more treatments in the various assays. However, only free arginine levels were significantly altered by all four treatments: water deficit, shade, weed competition, and application of kaolin particle film.

DISCUSSION

FAA levels peculiar to the different treatments appear to represent stress-specific FAA profiles for some cotton varieties and possibly other cotton varieties and other species of plants. Showler et al. (1991) demonstrated that late-season weedy habitats were associated with changes in 16 FAAs in sugarcane stalks. The same study showed that soil-incorporated aldicarb caused a significant change in free proline, and addition of phytophagous nematodes (*Criconemella*, *Meloidogyne*, *Paratrichodorus*, *Pratylenchus*, *Rotylenchulus*, and *Helicotylenchus* spp.) to

sugarcane plots resulted in differences in free cysteine, histidine, proline, and serine accumulations. Similarly, virus-induced stress in sugarcane, for example, was associated with reductions in nine FAAs (Showler et al., 1990).

Water Deficit Stress. The higher levels of some FAAs, especially proline, in association with water deficit stress is consistent with findings in other plants, including soybeans, *Glycine max* (L.) Merr. (McQuate and Connor, 1990); wheat, *Triticum aestivum* L. Pers. (Gusev and Gordon, 1968); sugar beets, *Beta vulgaris* L. (Gzik, 1996); maize, *Zea mays* L. (Ranieri et al., 1989); cowpea, *Vigna unguiculata* L. (Labanauskas et al., 1981); tomato, *Lycopersicon esculentum* Mill. (Rhodes et al., 1986); and jack pine, *Pinus banksiana* Lamb. (Cyr et al., 1990). In corn seedlings, drought treatment caused an increase in total FAA content and a consistent rearrangement of the amino acid pool with increased accumulation of free alanine, arginine, valine, aspartate, serine, threonine, and tyrosine. The lower amounts of free glutamic acid, glycine, and methionine likely resulted from a strong decrease in glutamate.

Rhodes et al. (1986) exposed suspension culture cells of water-deficit-adapted tomato to polyethylene glycol and found a 300-fold elevated free proline pool, primarily the consequence of a 10-fold rise in proline synthesis via the glutamate pathway. Using ^{15}N labels, they determined that ornithine was an unlikely precursor for proline and suggested that the rate of proline synthesis in unstressed cells only slightly exceeds the rate required to sustain both protein synthesis and proline pool maintenance with growth. Mechanisms restricting free proline oxidation and oxidation of other FAAs can operate at different rates governed by the degree to which water deficit stress is imposed on cotton.

Fernandes (1991) interpreted the accumulation of large pools of free proline and other FAAs in water-deficit-stressed rice as being an indication of disruption in the pathway to synthesis, or increase in the degradation of proteins. Chiang and Dandekar (1995) concluded that the higher free proline levels in *Arabidopsis thaliana* (L.) Heynh tissues with low water content resulted from increased biosynthesis. Barnett and Naylor (1966) determined that water-deficit-stressed *C. dactyla* accumulated 10 to 100 times more free proline than the controls and suggested that because proline turns over more slowly than other FAAs during water deficit stress, it may function primarily as a storage compound. Alternatively, Bussis and Heineke (1998) found that hydroponic potatoes subjected to water deficit stress induced by polyethylene glycol were able to grow in dry conditions by accumulating organic solutes for use in osmoregulation. Osmoregulation in the tubers occurred in two phases: during the first two days hexoses were accumulated, and after seven days, osmotic adjustment was due mostly to the accumulation of FAAs, especially proline (150 times the amount in unstressed controls). Osmotic stress in *A. thaliana* induced a rapid accumulation of free proline through *de novo* synthesis from glutamate, and during recovery from osmotic stress, accumulated free proline was oxidized to glutamate and the first step of

this process was catalyzed by proline oxidase (Verbruggen et al., 1996). Golan-Goldhirsh et al. (1989) suggested that free proline acts as a compatible solute, a readily utilizable source of energy, and a sink for soluble N. They also indicated that free proline accumulation in plant cells under osmotic stress is not the result of damage, but accompanies survival and growth, and that plants originating in dry climates tend to have higher levels of free proline than plants in wetter climates. Gadallah (1995) demonstrated that proline sprayed on cotton plants counteracted the effects of water deficit stress and significantly increased chlorophyll content, chlorophyll stability to heat, leaf relative water content, and dry matter content. FAA accumulations associated with disease and nematode infections of plants might result from osmotic disruption, but also for other reasons, including xylem injury repair (Cohn and Orion, 1970), syncytial wall formation of nematode-induced root galls (Lewis and McClure, 1975), cell wall formation (Epstein and Cohn, 1971), and blockage of protein synthesis (Chowdhury et al., 1985).

Diffusive resistance reflected the rates of free proline accumulation. In cotton, as in other plant species, free proline levels appear to indicate water deficit stress. In both water deficit assays, elevated concentrations of arginine, histidine, and valine were detected at the highest stress levels, which suggests that FAA "profiles" might be characteristic of certain kinds of stress in cotton foliage. Competition with alfalfa seedlings resulted in a 2.9-fold increase ($P \leq 0.05$) in free proline and lower transpiration; both are characteristics of water deficit stress in plants (Rhodes et al., 1986).

The differences in FAA concentrations and diffusive resistances in cotton grown on the 1000 ml/week irrigation regime in parts 1 and 2 might have resulted from differences between seasons when the two assays were run. The changes in rates of accumulation with increasing water deficit observed in part 2 supports other findings that show nonlinear FAA accumulations with increasing levels of stress (Parrott et al., 1969). The significant difference only between the sets of the two driest and two wettest treatments demonstrates that leaf water potentials dropped with increasing conditions of water deficit. However, changes in free proline levels did not occur at corresponding rates. On the surface, it appears that free proline might not influence osmoregulation, but drawing firm conclusions based on correlating water potential measurements taken from other plants at a different time of year is difficult.

Shade Assay. The taller cotton plants under shade conforms to the findings of Ballaré and Casal (2000). Makus (1993) found that protein N in *Amaranthus* sp. was higher in conditions of reduced light, possibly enabled by accumulations of free arginine. Durzan (1971) found that free arginine in jack pine and spruce was higher in shade, and orthogonal comparisons over shade indicated a highly significant fit of arginine N with increased shade. Reduced free aspartic acid in combination with higher amounts of free arginine agrees with the trends

TABLE 8. RATIOS OF FAA CONCENTRATIONS IN WEED-FREE COTTON:WEEDY COTTON AND COTTON IN FULL DAYLIGHT:SHADED COTTON

	Ratios[a]	
	Weed-free	No shade
FAA	weedy	shade
Alanine	1.04	0.85
Arginine	0.08[a]	0.49[a]
Aspartic acid	1.20[a]	1.23[a]
Glutamic acid	1.19[a]	1.02
Glycine	0.72	1.02
Histidine	0.14[a]	1.25
Isoleucine	0.57[a]	1.32
Phenylalanine	0.46[a]	1.02
Proline	1.02	1.23
Serine	1.12	1.02
Threonine	1.22[a]	0.98
Valine	1.10	0.80
Total essential FAAs	0.65[a]	0.92
Total FAAs	0.18[a]	1.05

[a] $P \leq 0.05$ between weedy and weed-free, and between shaded and unshaded; weed competition assay, $n = 15$, shade assay, $n = 20$.

shown in the weed competition assay (Table 8). The observation in this study that unbound arginine was the only source of detectable FAA N to increase suggests that shade probably increased protein N rather than FAA-N. Free proline levels were not altered by weed competition or shade, which indicates neither stress involved water deficit. The relatively small (≈7%) difference between leaf water potentials indicates that water deficit stress is not reducing relative water content of the foliage. Being that the weed competition assay was conducted in field plots and the shade assay occurred in a greenhouse, other environmental parameters (e.g., pest damage, sunburn, herbicide residual, competition for root space, nutrient deprivation) might have acted singly or in combination to cause seven FAAs other than arginine and aspartic acid to accumulate to different levels in the field plots. The smaller increase of free arginine grown in 50% reduced daylight as compared to the larger increase in the weedy cotton foliage might be further evidence of the influence of other stresses on free arginine under field conditions.

Arginine is synthesized in plants from ornithine, and it appears to serve as an endogenous source of N (Ludwig, 1993) and a substrate for protein synthesis (Durzan, 1971). In dicots, such as cotton, increased stem growth in conditions of

shade is often observed (Morgan and Smith, 1970). Shargool et al. (1988) demonstrated that enzymes of arginine metabolism such as arginase and urease increase during seed germination, a time of rapid growth. Plants can use either ornithine decarboxylase or arginine decarboxylase to produce the polyamine precursor putrescine. Arginine and putrescine biosynthesis both occur in active photosynthetic tissues; the primary enzymes of the arginine pathway (from glutamate to citrulline) are chloroplastic, whereas the terminal step from citrulline to arginine is carried out in the cytoplasm (Borell et al., 1995). In cotton, shade decreased the photosynthesis rate but increased concentrations of chlorophyll (Zhao and Oosterhuis, 1998). Research on effects of shade on FAA levels is needed to demonstrate the physiochemical cause for the association of shade with elevated free arginine levels in cotton foliage.

Weed Competition Stress. Shainsky and Rose (1995), studying Douglas fir, *Pseudotsuga menziessi* [Mirb.] Franco, and red alder, *Alnus rubra* [Bong.], found that competition among plants accounted for 44% of the variation in FAA accumulations. They concluded that FAAs, storing surplus N in light-limited plants, increased as a result of competition. This study showed that some competition with alfalfa seedlings for finite water may have caused a 2.9-fold increase ($P < 0.001$) in proline over cotton grown without alfalfa seedlings. However, the lack of significant difference in free proline levels indicated that in the weedy and weed-free plots, water deficit was not a cause of stress, and this was supported by the similar water potentials found in the two treatments. A primary difference between the cotton planted with alfalfa seedlings and the cotton grown with weeds is that the former did not cover any of the cotton canopy whereas the weed canopy cast shade on the cotton canopy throughout each day. The primary difference in terms of FAAs was the 12.4-fold elevation of accumulated arginine in the weedy cotton. In addition to being a precursor to proline (Shargool et al., 1988), arginine is thought to serve under some conditions as a store of N and for biosynthesis of secondary products such as polyamines (Ludwig, 1993). Shading, whether caused by other plants or nonvegetative structures, causes increased upward growth (Morgan and Smith, 1970; Ballaré and Casal, 2000), which requires polyamines for protein construction. However, the shorter cotton in the weed competition plots as compared to the taller shaded cotton in the greenhouse appears to be a result of thigmomorphogenesis induced by the mechanical perturbation of wind (Jaffe and Forbes, 1993). Nutrient depletion and rhizosphere restriction might have also contributed to lower field cotton heights.

The lower transpiration rates in weedy cotton agrees with the effects of both water deficit and light reduction in plants (Zhao and Oosterhuis, 1998), and weeds can conceivably cause each or both conditions. These findings might help to explain the FAA levels detected in another crop. Showler et al. (1991) found less free proline and free arginine (45% and 55%, respectively) in sugarcane stalks. The samples were taken in late October, after the weed growth had died and formed a

dense mat that retarded water evaporation from the soil. In the weed-free habitats where soil moisture could evaporate more freely, free proline levels were higher. Weed-free sugarcane stalks had more free arginine, possibly because weed pressure had earlier reduced the sugarcane stand density (Showler and Reagan, 1991). The more dense sugarcane growth in the weed-free regimes probably resulted in greater shading from other sugarcane plants.

Kaolin Particle Film Effects. Free proline accumulations were unaffected by kaolin, so the particle film did not induce water deficit stress to the cotton, and this conclusion was corroborated by the lack of significant difference among the leaf water potentials observed in each treatment. Soybeans dusted with kaolin had lower transpiration rates and used less water as a result of increased global and long-wave radiation reflection and reduced emissivity of kaolin-treated leaves (Baradas et al., 1976). Stanhill et al. (1976) showed that grain sorghum and cotton treated with kaolin used less water than untreated plants. Heightened light reception or photosynthetic activity might be associated with free arginine reductions in foliage coated with kaolin. However, Makus (2000) did not find a difference in cotton canopy light intensity as a result of kaolin application. Glenn et al. (1999) found that kaolin application (up to 3000 μg/cm^2 leaf surface) did not reduce apple, pear, and peach leaf photosynthetic activity, but neither did they report any increase.

Whether photosynthesis was enhanced by kaolin sprays or not, there was a reduced need for free arginine. If free arginine serves as an endogenous source of N, then the smaller pools of free arginine suggest that the plant was building nitrogenous molecules using more alternative sources of N. Accumulation of free arginine in the absence of elevated levels of free proline appears to be an indicator of shade stress, much like elevated free proline levels indicate water deficit stress.

Although transpiration rate and diffusive resistance reflected levels of water deficit stress, the accuracy of the porometer is heavily influenced by cloud cover, relative humidity, and wind speed (Idso et al., 1987). Quantification of FAAs is more complex and time-consuming than porometry, but it circumvents those parameters that can cause spurious variation in porometer measurements, and permits stress assessment when plant stomates are either open or closed. Furthermore, porometry alone was not useful in distinguishing between light reduction and water deficit stress effects.

This study shows that characteristic associations between selected FAA levels and stress factors were useful in analyzing stress levels associated with weed competition and foliar coverage with kaolin. It also shows that total detectable free essential amino acid accumulations were significantly heightened by water deficit and weed competition stresses, whereas kaolin-treated cotton had significantly less total free essential amino acids than controls. The beet armyworm, *Spodoptera exigua* (Hübner), has been reported to oviposit more on stressed cotton (Ruberson et al., 1994) possibly because free essential amino acid content is higher (Showler 2001b) in water stressed plants. Research focusing on physiochemically mediated

relationships between plant stresses and phytophagous pests might provide new approaches to developing plant resistance and tolerance to some pests.

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ANNOUNCEMENT

19TH ANNUAL MEETING OF THE INTERNATIONAL SOCIETY OF CHEMICAL ECOLOGY AUGUST 3–8, 2002, HAMBURG

The 19th annual ISCE meeting will be held at the Department of Chemistry, University of Hamburg, Germany. The conference will begin on the evening of August 3, 2002 (Saturday) and end on the evening of August 7 (Wednesday). The meetings will be hosted by Wittko Francke.

There will be four symposia covering aspects of chemical ecology in symbiosis, molecular biology, insect-plant relationship, and pheromone chemistry.

Both oral and poster presentations are invited from participants. Contributed papers fitting to the symposia are particularly welcome.

Participants are encouraged to arrive on Saturday, August 3, and attend the Social Lecture given by Prof. Dr. Jerrold Meinwald followed by a mixer that evening. The concluding banquet will be held Wednesday evening, August 7.

Further Information may be obtained on the meetings web site http://isce2002.chemie.uni-hamburg.de

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CALL FOR PAPERS

SPECIAL ISSUE ON AQUATIC CHEMICAL ECOLOGY

There will be a special issue of the *Journal of Chemical Ecology* devoted entirely to papers dealing with "Aquatic Chemical Ecology." Publication is planned for late summer/early fall 2002. The issue will consist of invited mini-reviews and contributed papers. Those wishing to submit a paper for consideration for this special issue should do so by March 15, 2002.

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STRUCTURE–ACTIVITY RELATIONSHIPS OF PYRROLIZIDINE ALKALOIDS IN INSECT CHEMICAL DEFENSE AGAINST THE ORB-WEAVING SPIDER *Nephila clavipes*

KARINA LUCAS SILVA and JOSÉ ROBERTO TRIGO*

Laboratório de Ecologia Química
Departamento de Zoologia
Instituto de Biologia, UNICAMP
Caixa Postal 6109, 13083-970, Campinas, SP, Brazil

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Abstract—Pyrrolizidine alkaloids (PAs) are known to protect Arctiidae moths and Danainae and Ithomiinae butterflies against the orb-weaving spider *Nephila clavipes* (Araneae, Araneidae, Tetragnathinae), which liberates adults of these insects unharmed from its web. We tested against this spider the role of eight PAs and one derived structure [an 89:11 mixture of the 12-membered macrocyclic diester free base integerrimine and senecionine and the respective N-oxide; two hydrolysis products from this mixture (the necine base retronecine, its respective N-oxide, and a mixture of integerriminic and senecionic necic acids); the 12-membered macrocyclic senkirkine; the 9-*O*-monoester free base senecioylretronecine and its respective N-oxide; and the 9-*O*-monoester free base callimorphine (a PA biosynthesized only by insects from retronecine)]. The mixture integerrimine–senecionine N-oxide seems to be more active than the respective free base [$LibD_{50}$ (liberation dose 50) = 0.042 and 0.153 μg/dry weight of prey, respectively], but the difference in activity between the N-oxide and free base of the 9-*O*-monoester senecioylretronecine was slight ($LibD_{50}$ = 0.167 and 0.104, respectively). Senkirkine, an otonecine base PA that does not form N-oxide and is not found in insects, was the less active, showing the highest $LibD_{50}$ (0.354). The difference in antipredator activity between N-oxides and free bases from macrocyclic diesters and monoesters may be correlated with physicochemical properties of these molecules in interaction with the *Nephila* receptors. For the active structures, there was a significant correlation between dosage and antipredator activity. Both forms of retronecine and a necic acid mixture were inactive, supporting the hypothesis that PAs biosynthesized by insects from retronecine were originally produced and stored in order to optimize chemical defense. Comparison of dose/activity data with reported amounts of

*To whom correspondence should be addressed. E-mail: trigo@unicamp.br

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PAs in butterflies suggested that, in general, PA-specialist insects are protected against predation by *Nephila*.

Key Words—Senecionine, integerrimine, retronecine, senkirkine, senecioyl-retronecine, callimorphine, *Nephila clavipes*, N-oxide, free base, sequestration, chemical defense, dose–activity, Araneae, Tetragnathinae, pyrrolizidine alkaloids.

INTRODUCTION

Chemical defense against predators due to storage of plant secondary compounds occurs widely in insects (see Brower, 1984; Bowers, 1990; Brown and Trigo, 1995; Hartmann and Witte, 1995; Hartmann and Ober, 2000; Trigo, 2000 and references therein). In a classical study, Brower et al. (1967) verified that adults of the monarch butterfly, *Danaus plexippus* (Nymphalidae, Danainae), were protected against predation by birds due to cardenolides sequestered from their larval host plants *Asclepias* (Asclepiadaceae). Other compounds active against predation were later found to be sequestered from host plants by insects, including iridoid glycosides, quinolizidine alkaloids, aristolochic acids, diterpenes, pyrazines, and pyrrolizidine alkaloids (PAs).

The defensive activities of PAs against predators have been studied since 1980 and are demonstrated against invertebrate and vertebrate predators such as spiders (Eisner, 1982; Brown, 1984, 1985, 1987; Masters, 1990; Eisner and Eisner, 1991; Trigo et al., 1993, 1996a; Orr et al., 1996; Gonzalez et al., 1999), ants (Dussourd et al., 1988; Hare and Eisner, 1993), lizards (McLain and Shure, 1985; Masters, 1992), and birds (Cardoso, 1997; Masters, 1992). The activity against visually oriented predators may be involved in the evolution of aposematic coloration of Ithomiinae, Danainae, and Arctiidae (Brower, 1984).

The most studied PA–prey–predator system involves the liberation of PA-protected arctiid moths and ithomiine and danaine butterflies by the orb-weaving spider *Nephila clavipes*, a Neotropical spider found in forest gaps and corridors, which are flight paths for insects (Robinson and Mirick, 1971). *N. clavipes* preys on bees, wasps, moths, and butterflies, but discriminates aposematic Ithomiinae and Danainae butterflies and Arctiidae moths, liberating them unharmed from the web (Belt, 1888, p. 317; Vasconcellos-Neto and Lewinsohn, 1984). Eisner (1982) was the first to show that PAs were responsible for liberating an insect (the arctiid *Utetheisa ornatrix*) without harm by *N. clavipes*. Other authors confirmed this chemical role (Brown, 1984, 1985, 1987; Masters, 1992; Trigo et al., 1993, 1996a). No studies were done on PA structure and dosage versus activity in relation to *N. clavipes* (but see Masters, 1992 for dose–activity relations in vertebrate predators).

In this paper, we report experiments to test the relative activity of some different types of alkaloids against *Nephila clavipes*. The questions addressed in the

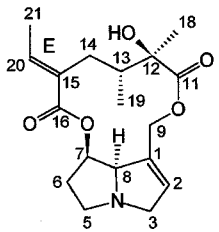
PAs to be bioassayed were: (1) Since N-oxides are the main PAs in plants and insects, and the latter have the capacity to N-oxidize dietary free bases (Hartmann and Witte, 1995), would there be difference in their activity? In order to ascertain this, we bioassayed a 1,2-unsaturated 12-membered macrocyclic diester, a 9-*O* monoester, their respective N-oxides, and also an otonecine 12-membered macrocyclic diester that does not form an N-oxide. (2) What chemical functions in the molecules give activity to PAs? Would insect PAs be biosynthesized to optimize chemical defense? To answer these questions, we bioassayed, in addition to the above PAs, the hydrolysis products from the first (a necine base and its respective N-oxide, and necic acids), and callimorphine free base, an insect PA biosynthesized from retronecine by several arctiid moths (Hartmann and Witte, 1995).

METHODS AND MATERIALS

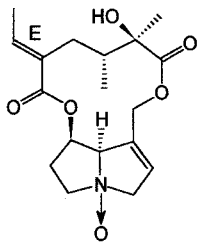
Sources of PAs. We used for the bioassays free bases and N-oxides of a mixture (89:11) of integerrimine and senecionine [henceforward ISFB (Figure 1, **1a**) and ISNO (**1b**)], senkirkine [SKK (**2**)], the necine base, retronecine [RTFB (**3a**) and RTNO (**3b**)], the mixture of necic acids, integerriminic:senecic acids [ISAC (**4**)], the free base and N-oxide of 9-*O*-senecioylretronecine [SRFB (**5a**) and SRNO (**5b**)], and callimorphine free base [CLFB (**6**)].

ISFB, with two geometric isomers at C_{20}–C_{21}, was isolated from aerial parts of *Senecio brasiliensis* L. (Asteraceae: Senecioneae) from southeast Brazil. These 1,2-unsaturated macrocyclic diesters, also isolated from this species by Hirschmann et al. (1987) and Klitzke and Trigo (2000), are characteristic of Senecioneae plants. They are sequestered by several arctiid moths for defense and as chemical communication precursors (see for reviews Brown and Trigo, 1995; Hartmann and Witte, 1995; Trigo, 2000). The alkaloids were extracted according to Trigo et al. (1996a,b) and chromatographed over silica gel, eluting with a gradient of $CHCl_3$–MeOH from 99:1 to 90:10. The resulting mixture of integerrimine–senecionine was analyzed by GC-MS (Witte et al., 1993), ^{1}H, ^{13}C, COSY and HETCOR NMR (300 and 500 MHz). The ^{1}H NMR spectral comparison was done following data from Segall and Dallas (1983), Roitman (1983), and Logie et al. (1994) for ISFB and from Urones et al. (1988) for ISNO [the N-oxidation methodology for all compounds followed Craig and Purushothaman (1970)]. The ^{13}C signals were compared with Roeder (1990) for both ISFB and ISNO.

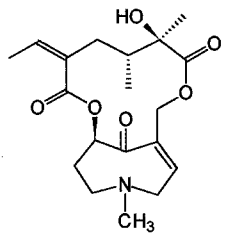
RTFB and ISAC were obtained by the hydrolysis of ISFB in a basic medium as previously described by Nishida et al. (1991). When necessary, RTFB was purified as above with $CHCl_3$–MeOH 3:1 over a basic alumina column. The structures were identified by the methods cited above. The ^{1}H NMR spectral comparison was done following data from Culvenor et al. (1965) for RTFB, Bohlmann et al. (1986) for RTNO, and Niwa et al. (1992, 1994) for ISAC. The ^{13}C signals for retronecine and



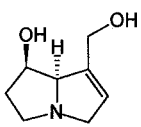
1a. Integerrimine:senecionine free base (ISFB) Senecionine = Z



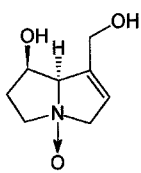
1b. Integerrimine:senecionine *N*-oxide (ISNO) Senecionine *N*-oxide = Z



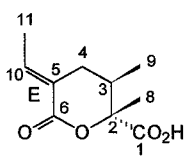
2. Senkirkine (SKK)



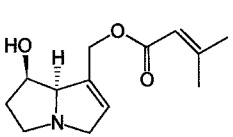
3a. Retronecine free base (RTFB)



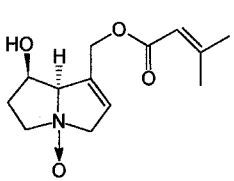
3b. Retronecine *N*-oxide (RTNO)



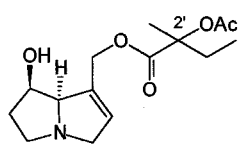
4. Integerriminic:senecic acids (ISAC) Senecic acid = Z



5a. 9-*O*-Senecioylretronecine free base (SRFB)



5b. 9-*O*-Senecioylretronecine *N*-oxide (SRNO)



6. Callimorphine free base (CLFB)

FIG. 1. Pyrrolizidine alkaloids and derivatives used for *Nephila clavipes* bioassays.

its N-oxide were compared with Roeder (1990), and for ISAC they were assigned by HETCOR and DEPT analysis.

SRFB was obtained by the coupling of RTFB with senecioic acid regioselectively at C-9, following methodology developed by Nishimura et al. (1987), using dichloromethane instead of toluene as solvent. CLFB was obtained by the same methodology, using instead of senecioic acid (±)-2-acetoxy-2-methylbutanoic acid, synthesized from (±)-2-hydroxy-2-methylbutanoic acid refluxed 24 hr in acethylchloride. We obtained CLFB as a mixture of diastereoisomers at the

C-2′ position (see Figure 1). The ^{1}H NMR spectral comparison was done following data from Bohlman et al. (1986) for SRFB and SRNO, and L'Empereur et al. (1989) for CLFB. The ^{13}C signals for CLFB were also compared with L'Empereur et al. (1989), and for SRFB and SRNO they were assigned by HETCOR and DEPT analysis.

Bioassays with Nephila clavipes. The experiments with female *Nephila clavipes* (L.) (Araneae, Tetragnathinae) were carried out from March to May, in 1995, and in 1997–2000 at two sites: Serra do Japí, Jundiaí, São Paulo, and Instituto Agronômico de Campinas, Campinas, São Paulo. All experiments were performed between 09:00 and 16:00 hr. Only healthy female spiders were used, that is, spiders that responded immediately and in their typical way (Robinson and Mirick, 1971) when any prey was tossed onto their web. Spiders already feeding on any prey were not used. Each spider was used only once a day.

Workers of *Apis mellifera* L. (Apidae), with their stings intact, were killed by freezing, had their wings removed, and were used as carriers of tested PAs in the bioassays. Ten microliters of different amounts of the compounds soluble in MeOH [6.50×10^{-4}, 2.61×10^{-3}, 1.04×10^{-2}, 4.18×10^{-2}, 1.67×10^{-1}, 6.68×10^{-1}, and 2.67 μmol/bee or 3.82×10^{-5}, 1.54×10^{-4}, 6.14×10^{-4}, 2.46×10^{-3}, 9.82×10^{-3}, 3.93×10^{-2}, and 1.57×10^{-1} μmol/mg dry weight of bee (mean dry weight 17 mg)] were coated topically onto each bee with a microsyringe. The concentrations were based on the mean amount of PAs in Ithomiinae butterflies ($\approx$200 μg or 6.68×10^{-1} μmol; see Brown, 1985) and increased (once) or decreased (five times) in a geometric ratio of 4. ISAC was bioassayed only at the highest concentration. The bee was offered to the spider, whose response in relation to the prey was recorded (prey or liberate, see also Robinson and Mirick, 1971; Vasconcellos-Neto and Lewinsohn, 1984). When the spider preyed on the alkaloid-treated bee, the trial was finished. If the spider liberated a bee, another one treated with 10 μl of pure MeOH was offered immediately and the trial was valid only if the spider preyed on it. Each amount of alkaloid or derivative was tested at least 10 times.

Statistical Analyses. The 50% liberation dosages ($LibD_{50}$) were calculated by the linear regression of the probit of the percentage of liberated individuals *versus* log of micromoles per milligram of dry weight of the bees (Finney, 1971; Haddad, 1986). The regression lines were compared using analysis of covariance (ANCOVA) with a post hoc Tukey test (Sokal and Rohlf, 1995).

RESULTS

Neither RTFB nor RTNO showed activity against predation by *N. clavipes*: no dosage incited 50% liberation by spiders. At high concentration (3.93×10^{-2} μmol/mg), these compounds showed around 20% liberation (Table 1). ISAC

TABLE 1. NUMBER OF LIBERATED PREY INDIVIDUALS PER TOTAL BIOASSAYED PREY FOR PAS TESTED IN *Nephila clavipes* BIOASSAY[a]

Concentrations (μmol/mg dry weight of prey)	PAs[b]								
	ISFB (**1a**)	ISNO (**1b**)	SKK (**2**)	RTFB (**3a**)	RTNO (**3b**)	ISAC (**4**)	SRFB (**5a**)	SRNO (**5b**)	CLFB (**6**)
3.82×10^{-5}	6/30 (20)	9/30 (30)	0/11 (0)	1/30 (3.3)	1/30 (3.3)	NT	4/30 (13.3)	5/30 (16.7)	0/10 (0)
1.54×10^{-4}	5/30 (16.6)	**15/30 (50)**	2/10 (20)	1/30 (3.3)	2/30 (6.7)	NT	7/30 (23.3)	12/30 (40)	1/14 (7.1)
6.14×10^{-4}	4/10 (40)	**7/10 (70)**	6/13 (46.2)	0/11 (0)	0/12 (0)	NT	**18/30 (60)**	8/30 (26.7)	3/9 (33.3)
2.46×10^{-3}	**27/20 (90)**	**30/30 (100)**	**10/11 (90.9)**	0/30 (0)	1/30 (3.3)	NT	**23/30 (76.7)**	**18/30 (60)**	**11/14 (78.6)**
9.82×10^{-3}	**8/10 (80)**	**30/30 (100)**	**12/16 (75)**	0/10 (0)	0/10 (0)	NT	**15/15 (100)**	**28/30 (93.3)**	NT
3.93×10^{-2}	**30/30 (100)**	**30/30 (100)**	**13/14 (92.8)**	7/30 (23.3)	5/30 (16.7)	1/12 (8)	**30/30 (100)**	**30/30 (100)**	NT

[a] The numbers in parentheses represent the percent liberated. NT = not tested. The concentrations that elicited ≥50% of liberation by the spider are shown in bold.

[b] ISFB = integerrimine–senecionine free bases; ISNO = integerrimine–senecionine N-oxides; SKK = senkirkine; RTFB = retronecine free base; RTNO = retronecine N-oxide; ISAC = integerriminic: senecionic acids; SRFB = 9-*O*-senecioylretronecine free base; SRNO = 9-*O*-senecioylretronecine N-oxide; CLFB = callimorphine free base.

TABLE 2. LIBERATION DOSE 50 ($LibD_{50}$) OF PAS IN *Nephila clavipes* BIOASSAYS

PA	Concentration		Regression lines	P^a
	μmoles/mg dry weight prey	μg/mg dry weight prey		
ISFB (**1a**)	5.0×10^{-4}	0.153	$y = 1.121x + 8.744$	a
ISNO (**1b**)	1.0×10^{-4}	0.042	$y = 1.406x + 10.516$	b
SKK (**2**)	1.0×10^{-3}	0.354	$y = 1.032x + 8.110$	c
SRFB (**5a**)	4.0×10^{-4}	0.104	$y = 1.251x + 9.199$	a,d
SRNO (**5b**)	7.0×10^{-4}	0.167	$y = 0.937x + 7.979$	a,c,d

[a] ANCOVA: the slopes had no significant differences; different letters denote significant differences in the intercept at 5% level.

also did not have antipredator activity. At the highest concentration tested (3.93×10^{-2} μmol/mg), only 1 of 12 preys was liberated by *N. clavipes*; due to the inactivity of this mixture, bioassays with smaller concentrations were not done (Table 1).

The other six bioassayed PAs were effective against predation by *N. clavipes*; all of them had at least one concentration that elicited 50% prey liberation by the spider (Table 1). For five, the $LibD_{50}$ was calculated, decreasing in the following order: ISNO > SRFB > ISFB $\cong$ SRNO $\gg$ SKK (Table 2); it is interesting to note that SRFB had a higher activity than SRNO. For CLFB, no $LibD_{50}$ was calculated since the synthetic yield was very low (around 20%), and there was not enough material to carry out more bioassays. At a concentration of 2.46×10^{-3} μmol/mg, 80% of the treated bees were liberated (Table 1); this is similar to the results obtained for SRFB and higher than those for SRNO (Table 1).

The line regression statistical analysis by ANCOVA showed that all five analyzed PAs had no significant differences in the slopes, and the intercept of ISNO was significantly higher than the others, representing the highest activity against predation (Table 2). SKK showed the lowest intercept, representing the lowest activity (Table 2).

DISCUSSION

Are There Differences between the Activity of N-Oxides and Free Bases? Our data showed that, for the macrocyclic diester mixture IS, the N-oxide was more active than the free base, but for senecioylretronecine it was not. The high activity of N-oxide in relation to its respective free bases was also found by other authors. Dussourd et al. (1988) topically applied monocrotaline in its free base and N-oxide forms on alkaloid-free eggs of *Utetheisa ornatrix* and observed that the latter was more active against predation by the beetle *Coleomegilla maculata* (Coccinellidae). Hare and Eisner (1993) repeated these bioassays against the ants *Leptothorax longispinosus* and found the same result. Moreover, ants with previous

contact with N-oxides preyed upon fewer alkaloid-free eggs in subsequent trials than ants with previous contact with free bases. However, Dreyer et al. (1985) found ambiguous results: senecionine N-oxide applied in a diet was 1.7 times more active than the respective free base against feeding by the pea aphid *Acyrthosiphon pisum*, but for riddelliine and monocrotaline, an inverse response was found.

We propose that the activities of free bases and N-oxides against *N. clavipes* are closely related to their intrinsic physicochemical features in relation to the spider receptors. The macrocyclic diester IS has a more rigid conformation than the 9-*O*-monoester SR, with few degrees of freedom, restricting its conformational possibilities. The free rotation around the C_9-O_{10}-C_{11} links in SR allows more conformational possibilities. For IS, the N-oxide is more active than the free bases. We hypothesize that this might be related to a restricted conformation, which could activate more efficiently the O on the N. This activation could facilitate the interaction of PA–spider receptors better than in free bases where this activation could not occur. For SR, there seems to be no difference in antipredation activity between free bases and N-oxides. Again, we hypothesize that the high degrees of freedom in the ester linkage would promote a loss of specificity at the N site when it is N-oxidized. The speculation calls for bioassays with more different PA structures and chemical calculations for these molecules, such as *ab initio*, semiempirical, or molecular orbital methods, to obtain physical and chemical parameters for a QSAR analysis.

Senkirkine, a macrocyclic PA that does not form an N-oxide and has as necine base (otonecine) with an N-methyl group (Hartmann and Witte, 1995), was less effective against predation by *N. clavipes*. Frei et al. (1992) found that senkirkine was more strongly genotoxic to *Drosophila melanogaster* than senecionine. This alkaloid is a more derived PA in plants, along the same biosynthetic pathway as senecionine (Hartmann and Dierich, 1998). These authors suggest that the diversity of structures formed from senecionine N-oxide, such as senkirkine, offers an excellent strategy for plant defense, increasing the escape success from local herbivory by generating populations with more deterrent or unpalatable PAs. However, nothing is known about insect incorporation of senkirkine, and its role as a defensive compound in this taxa; we can speculate that maybe the spider receptors are not able to recognize it.

What Chemical Functions in the Molecules Give Activity to PAs? Both retronecine and its N-oxide were ineffective against predation by *N. clavipes*. Dreyer et al. (1985) also found that retronecine was ineffective for deterrence against pea aphids and suggested that the function ester might be necessary for PA biological activity. More evidence for the importance of the ester function is that the acid mixture ISAC did not have activity against predation by spiders when tested alone. Moreover, specialist PA-sequestering butterflies have the capacity to produce specific insect PAs linking a plant necine base (probably formed by hydrolysis of ester PAs in insects) with a necic acid biosynthesized by the insect, which already

possesses an ester function. The insect PA activity in chemical defense is not clear, but we hypothesize that the biosynthesis of specific alkaloids could be an optimization of PA use in chemical defense against predators. Thus, the cost of sequestration of retronecine, ineffective against predation, could be compensated for by the biosynthesis of specific insect PAs, such as callimorphine, that protected insects against predation by *N. clavipes* better than RT. Hartmann et al. (1990) suggested that the production of insect PAs is linked to pheromone production by males, since the metabolic pathway to produce these involves hydrolysis of plant ester alkaloids. Nevertheless, since both males and females seem to produce these alkaloids (Hartmann et al., 1990), more studies must be done to clarify the biosynthetic pathway.

Unpalatability Spectrum Involving PAs. For six PAs bioassayed in this work, there was a positive correlation between dosage and antipredator activity against *N. clavipes.* Within any insect population or species, the degree of individual unpalatability can vary (Brower et al., 1968; Bowers, 1981). The chemical content of plants could be a critical determinant of the degree of protection conferred to insects that sequester host plant chemical compounds (Ritland, 1994). In an unpalatable insect population, the result of this chemical variation is a palatability spectrum (Brower et al., 1968; Brower, 1984). This phenomenon also results in automimicry as described by Brower et al. (1967), when more palatable insects are perfect mimics of less palatable individuals of the same species.

Nephila clavipes, like other predators, could test many individual potential prey items and reject the less palatable ones. Variation in defensive chemical quantity could determine which individuals would be killed and eaten and which would be liberated alive, as has been shown to occur with monarch butterflies, *Danaus plexippus* (containing different concentrations of cardenolides) in relation to the predator black-backed oriole, *Icterus abeillei* (Fink and Brower, 1981). These predators could act as selective agents on individual chemical variation. The results found in this work confirm this; prey with lower PA dosages were relatively more accepted than prey treated with higher dosages.

Brown (1985) determined the variation in PA concentration in 41 Ithomiinae species in the field. The range varied from 3 to 77 μg/mg of dry weight. All of these presented a higher average PA amount than that necessary to promote at least 50% liberation by *N. clavipes.* These data suggest that Ithomiinae butterflies in the field are protected against predation by *N. clavipes,* even though these butterflies present PA mixtures with structures different from IS, normally a mixture of intermedine and lycopsamine (Brown 1985, Trigo et al., 1996a,b). The data in Vasconcellos-Neto and Lewinsohn (1984) confirm this hypothesis, since *N. clavipes* liberated alive almost all Ithomiinae and Danainae butterflies offered to them. Our data showed that the PA amount that protects butterflies from predation by *N. clavipes* could be relatively low. The remaining PA found in butterflies could be used for other functions: in males for pheromone biosynthesis and a gift to females during

mating, and in females for transfer to eggs (Brown 1984, 1985, 1987; Trigo and Brown, 1990).

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TEMPORAL MODULATION OF PYRROLIZIDINE ALKALOID INTAKE AND GENETIC VARIATION IN PERFORMANCE OF *Utetheisa ornatrix* CATERPILLARS

KATHERINE C. KELLEY,* KELLY S. JOHNSON, and MITZI MURRAY

Department of Biological Sciences
Irvine Hall, Ohio University
Athens, Ohio 45701

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Abstract—Foraging on a variety of different food items allows individuals to balance nutrient intake, but it also may dilute toxins present in single items of the diet. The distinction between nutrient balancing and toxin dilution becomes less clear, however, for organisms that sequester potentially toxic compounds for their own benefit. Additionally, specific dietary components chosen may or may not affect metabolic rate, growth, and other measures of fitness. Using the arctiid moth *Utetheisa ornatrix*, we (1) evaluated the temporal pattern of larval feeding behavior when presented with diet enriched with the pyrrolizidine alkaloid (PA), monocrotaline, (2) estimated the cost of ingesting PAs by measuring larval nutritional performance on diets containing different amounts of usaramine, and (3) investigated the magnitude of genetic effects on the performance of families raised on diets of different usaramine concentrations. We demonstrate that an herbivorous insect can temporally vary its intake of sequestered allelochemical. Individuals combined a preference for intermediate amounts of monocrotaline with temporal modulation of the specific amount of PA ingested on a given day. Using usaramine, we found little evidence for a cost of ingesting PAs, except among some family groups. Measures of digestive and metabolic efficiency showed no consistent reduction in performance on higher alkaloid diets. Only when larvae in family groups were fed a single concentration of PA over more than one instar was there any evidence for a cost of ingestion. Additionally, there were large genetic components to both growth and an individual's growth response to dietary alkaloid. These results suggest that while genetic variation in performance on alkaloid-rich diets exists, modulation of intake rate by caterpillars may be an important mechanism to reduce effects on the growth and fitness of individuals.

Key Words—Pyrrolizidine alkaloid, larval diet choice, nutritional performance, larval growth, genetic effects, *Utetheisa ornatrix*, Arctiidae.

*To whom correspondence should be addressed. E-mail: csc11@oak.cats.ohiou.edu

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INTRODUCTION

Animals actively choose among different kinds of prey or food items during foraging (reviewed by Pyke et al., 1977; Stephens and Krebs, 1986; Waldbauer and Friedman, 1991). This diet mixing may achieve some optimal nutritional balance [e.g., marine invertebrates: (Rapport, 1980; Pennings et al., 1993); insects (reviewed by Simpson and Simpson, 1990); vertebrates (Leeson and Caston, 1991; Dearing and Schall, 1992)]. Alternatively, a diverse diet may dilute the impact of ingested toxins (Freeland and Janzen, 1974; Westoby, 1978). Interestingly, these two hypotheses may not be mutually exclusive for organisms that sequester plant allelochemicals for their own benefit.

While a physiological cost of ingestion and sequestration of secondary compounds is usually assumed and only sometimes demonstrated (reviewed by Bowers, 1992; Camara, 1997), there also can be fitness benefits to acquiring plant compounds (Boppré, 1986, 1990; Eisner and Meinwald, 1987). For many sequestering insects, the benefit is limited to a reduction in mortality (e.g., Eisner, 1980; Brower et al., 1984). For Lepidoptera specializing on host plants with pyrrolizidine alkaloids (PAs), however, the sequestration of alkaloids has a positive impact on reproductive success as well (Conner et al., 1981; Eisner and Meinwald, 1987; Dussourd et al., 1988, 1989). For this group of insects, PAs simultaneously might be considered nutrients (dietary component increasing survivorship, fecundity, etc.) and toxins (dietary component reducing survivorship, fecundity, etc.).

If an alleolochemical can be simultaneously nutritive and toxic, then its intake might influence the foraging behavior of those herbivore specialists that sequester their host's defensive compound(s). In the process of foraging, these specialists should not rely on a simple decision rule that identifies the allelochemical as a stimulant or deterrent for feeding. A more complex self-regulation of allelochemicals might balance the rate of intake, which is important for avoiding a toxic dose of the compound, with the cumulative intake, which determines the potential benefit an individual might accrue from sequestration of the compound. While several strategies might be employed to balance these, no study has detailed both the rate (intake per day) and rhythm (temporal variation in rate) of foraging decisions of these organisms to illustrate some of the strategies that might be employed.

The ornate moth [*Utetheisa ornatrix* (L.), Arctiidae] is an herbivore specialist that sequesters pyrrolizidine alkaloids from its *Crotalaria* hosts. The species is found on both native *Crotalaria* species and introduced ones (*C. pallida*, previously *C. mucronata*, and *C. spectabilis*). The introduced plants contain usaramine and monocrotaline, respectively, as their primary alkaloids; neither contains the alkaloid of the other (Dussourd et al., 1988). The PAs of *Crotalaria* could reduce some components of *U. ornatrix* fitness because of their general biological activity (Hartmann, 1991), yet both usaramine and monocrotaline are known to have a significant and positive impact on adult reproductive success in *U. ornatrix* (Conner

et al., 1981). Thus, PAs might be simultaneously toxic and nutritive to the larval *U. ornatrix* that sequester them.

We tested whether larvae of *U. ornatrix* actively regulate their intake of the pyrrolizidine alkaloid, monocrotaline, through temporal modulation of feeding behavior. Additionally, we estimated the cost of ingesting PAs by measuring the performance of larvae reared on diets containing different amounts of usaramine. Finally, we investigated the magnitude of genetic effects on the performance of families raised on different diets. If there are substantial physiological costs to ingesting pyrrolizidine alkaloids, larvae should prefer diets that minimize their alkaloid intake. If there is only a minimal cost to processing the allelochemical, then larvae should prefer diets that maximize their alkaloid intake. Finally, finding a family effect on the performance of larvae reared on different diets would suggest genetic variation in the physiological ability of larvae to process alkaloids and an opportunity for selection on this trait.

METHODS AND MATERIALS

Quantification of Alkaloids in Seed Powder. To measure seed alkaloid content, we modified the field test of Culvenor and Fitzgerald (1963). Dried *C. pallida* seeds were ground to a fine powder. The powder (2.7 g) was extracted in 50 ml ammoniacal chloroform (6.71 ml NH_4OH in 993.29 ml chloroform) for several hours. The extract was filtered and partitioned with 6 ml 2 N H_2SO_4. For total alkaloids, zinc dust was added to a 2-ml aliquot of the H_2SO_4 layer for several hours to reduce the N-oxides. A second 2-ml aliquot provided a measure of free base PA. The amount of precipitate formed 24 hr after addition of 6 drops of silicotungstinic acid was compared (visual determination of cloudiness) to a standard curve generated using known quantities of monocrotaline (equivalent to 0.51, 0.55, 0.84, 1.1, 1.7, and 3.3% dry wt).

The total alkaloid content of the seed powder was estimated to be between 1.1 and 1.7% dry wt. The concentration of free base (as opposed to N-oxide) in the seed powder was 0.06–1.1%, or roughly half. Based on these data, the concentration of PA in the four seed powder diets prepared (see below) was 0.017–0.026, 0.066–0.10, 0.13–0.20, and 0.26–0.41%. For simplicity, we will use only the larger estimated value.

Two-Choice Trials with Usaramine-Containing Seed Powder. Newly hatched larvae were reared on an alkaloid-free wheat germ and soy protein-based artificial (control) diet for 8–12 days (i.e., to the fourth or fifth instar). Then, larvae were transferred to small individual containers with loosely fitting lids and offered equal quantities of two test diets. Test diets contained 0% (control), 1.5%, 12%, and 24% dry wt of seed powder and were offered as paired choices (0% vs. 0.026% PA, 0.026% vs. 0.20% PA, and 0.20% vs. 0.41% PA). Varying the amount of seed

powder in artificial diet has been employed as a means of manipulating PA intake in numerous studies of alkaloid sequestration and use in *Utetheisa* (e.g., Conner et al., 1981; Dussoud et al., 1988). The seed powder replaced equivalent portions of indigestible cellulose, so the nutrient and water contents of the test diets did not vary (except with regard to differences specifically associated with the seeds). Larval feeding preferences were determined by comparing the amount (wet wt) of each diet consumed over 24 and 48 hr periods. Consumption was calculated by subtracting the wet mass of uneaten diet from the initial of amount of diet offered. Control (uneaten) portions of diet, weighed before and after trials, showed that water loss was negligible (less than 4% after 48 hr) and did not differ by diet. Twelve to fifteen larvae per trial were assayed.

The amount of diet eaten by larvae in each of the two-choice trials was analyzed with ANOVA. Effects in the model included individual, diet, time (24 and 48 hr after initially offered), and the interaction between diet and time. A significant interaction term would indicate that the rate of consuming a given diet changes over time and, therefore, suggest some modulation of feeding behavior in response to the diets available. Analyses were conducted with the GLM procedure (SAS Institute, 1989).

Four-Choice Trials with Monocrotaline. Larvae were reared on control diet until they approached the molt to the final (fifth) instar. At the premolting stage (head capsule slipped and gut emptied), larvae were weighed and placed in individual plastic boxes with four equal-sized (wet mass) diet cubes containing 0%, 1%, 4%, and 8% dry wt monocrotaline (free-base form; Sigma Co.). The pH of these diets ranged from 4 to 7 and was similar to that of natural caterpillar diets (Schultz and Lechowicz, 1986; Johnson and Felton 1996). Field-collected *C. spectabilis* contain a range (2.3–5.4% dry wt.) of monocrotaline (Johnson et al., 1985), so 8% represents approximately twice the concentration encountered by larvae in the wild. Diet cubes in the box were haphazardly assigned to one of four positions. The amount consumed from each was monitored daily for four days. All diet cubes in the arena were replaced when at least 70% of any one of the four had been consumed. Trials were performed with 22 individual larvae in two test groups, to accommodate the availability of appropriately aged larvae.

The amount of four monocrotaline diets eaten by larvae over a four-day trial was analyzed both with a repeated measures and randomized complete block design ANOVAs. To evaluate day and diet effects on individual feeding behavior, a repeated measures ANOVA was conducted with test group and sex as the between-subject effects. The Huynh-Feldt epsilon estimate approached unity for some within-subject effects ($0.78 < \epsilon < 1.05$); therefore, only multivariate tests of hypotheses were considered (SAS Institute, 1989). To further evaluate diet preferences and changes over the four-day period, randomized complete block designs were employed. Two individuals with missing data had to be excluded from this analysis to make the designs complete. This enabled multiple-range tests

[Ryan-Einot-Gabriel-Welsh (SAS Institute, 1989)] among diets or days, while still accounting for the nonindependence of observations made on each individual.

The final analyses of the four-choice experiment investigated the relationship between food and monocrotaline intake over time. Preliminary linear regression was conducted to evaluate whether intake of food or monocrotaline on a given day depended on the amount ingested on the previous day. Because intake rates appeared to oscillate, similar analyses were made to evaluate the dependence of changes in feeding (or the difference between days) on the previous day's intake. Correlation analyses indicated that food and alkaloid intake were related, so multiple regression analyses were performed using the change in intake on a given day as dependent variables, and both food and PA intake from all previous days in the trial as predictor variables. In the multiple regression, stepwise addition of predictor variables was employed. The variance inflation factors (VIF) for all multiple regression equations were less than 5; therefore multicollinearity, or intercorrelation among the predictor variables, was not large enough to affect the analyses (Stevens, 1996).

Nutritional Performance Study with Usaramine-Containing Seed Powder. Standard nutritional budgets were calculated for larvae fed diet containing *C. pallida* seed powder (0%, 0.10%, and 0.41% PA). Caterpillars were reared on control diet to the premolt stage for the fifth instar (10–15 days). Larvae were weighed, housed individually, and provided with preweighed cubes of treatment diet (15 larvae per treatment). Food was replenished daily as needed. Uneaten diet and frass were collected and refrigerated every other day. For each individual, pupation date and weight on the third day after pupation were recorded, then sex was determined using gross morphology. All uneaten diet, frass, shed skins, and silk were dried to constant weight and weighed to the nearest 0.1 mg. Indices were calculated on a wet-weight basis.

To improve normality, all three nutritional indices were arcsin transformed, growth rate was square-root transformed, and frass production was natural-log transformed. No transformation improved the skewed distribution of duration to pupation, so the variable was used untransformed.

Two separate multivariate analyses of covariance (MANCOVA) were performed, one on the nutritional indices and another on the six variables that comprise these indices, to test for multivariate effects of the diet treatments, family, gender, and the interaction between family and diet with initial weight as a covariate. The first analysis was conducted to provide results comparable to similar studies, while the second was performed to identify the specific changes in nutritional performance that contributed to effects on the calculated indices. The multivariate analyses were followed by univariate analyses (ANCOVA) to examine which variables contributed to significant multivariate effects. In these analyses, a diet effect would indicate changes in nutritional performance as a result of the amount of seed powder in the diet, a family effect would provide evidence of a genetic basis for the

response of the particular performance variable, a gender effect would imply males and females have different nutritional performances, and a family × diet interaction would demonstrate genotypes responded differently to the diet treatments.

Significant effects of diet on performance variables were further examined with multiple comparisons of least squares means. Because the experiment was not designed to test for genetic effects on nutritional performance, the power of the family and family × diet tests was not high. Type III sums of squares were employed because of the unbalanced design.

Genetic Effects on Performance with Usaramine-Containing Seed Powder. In a larger study, measures of growth were analyzed for seven full-sib families. Up to 60 larvae from each family were reared on control diet until approximately the third instar, then transferred to individual containers with experimental diet containing 0%, 0.10%, or 0.41% PA. Food was replenished daily as needed, and individuals were put in fresh containers when frass accumulated or mold appeared. Each individual was weighed at the time of transfer and at pupation so weight gain and growth rate could be calculated. Families were composed of all individuals that reached pupation (mean = 45, range = 19–56).

Pupal weight, weight gained, and growth rate were analyzed with a MANCOVA using initial weight as a covariate. The main effects included in the model were family, diet, and the family × diet interaction. This model was similar to that used for the previous experiment, although sex was not included. Analyses were conducted with the GLM procedure with type III sums of squares employed because of the unbalanced design; mean squares (MS) are reported.

RESULTS

Diet Choice Trials. In each of the two-choice trials, larvae always preferred the diet with more alkaloid (0% vs. 0.026% PA: MS = 0.068, $F_{1,39} = 26.27$, $P < 0.001$; 0.026% vs. 0.20% PA: MS = 0.064, $F_{1,33} = 45.32$, $P < 0.001$; and 0.20% vs. 0.41% PA: MS = 0.150, $F_{1,39} = 31.06$, $P < 0.001$). In two trials, larvae reduced their consumption of diet with less seed powder during the second 24 hr period (diet × time interactions, 0% vs. 0.026% PA: MS = 0.012, $F_{1,39} = 4.74$, $P = 0.036$; and 0.20% vs. 0.41% PA: MS = 0.025, $F_{1,39} = 5.26$, $P = 0.027$).

The pattern of larval feeding in the four-choice trial with monocrotaline differed among days and among diets (Table 1). Larvae consumed more diet with 1% or 4% alkaloid than diets with 0% or 8% (MS = 26,423, $F_{3,283} = 5.30$, $P = 0.001$). While this trend of preference for intermediate diets was observed on all days individually, only on the first day was there a significant difference in larval feeding (data not shown). The repeated measures analysis also showed other effects on larval feeding. The two groups tested ate different amounts of alkaloid on the four days tested (day × group interaction) and marginally differed in their feeding

TABLE 1. REPEATED MEASURES ANOVA FOR AMOUNT OF FOUR MONOCROTALINE DIETS EATEN OVER A FOUR-DAY TRIAL

Source	*df*	Wilk's lambda	*F*	*P*
Within-subject effects				
Day	3	0.229	15.66	<0.001
Diet	3	0.524	4.23	0.025
Day × diet	9	0.255	2.60	0.097
Day × group	3	0.254	13.65	<0.001
Day × sex	3	0.910	0.46	0.715
Diet × group	3	0.762	1.45	0.271
Diet × sex	3	0.883	0.61	0.617
Day × diet × group	9	0.228	3.00	0.068
Day × diet × sex	9	0.142	5.36	0.014
Error	8, 14			
		MS		
Between-subject effects				
Group	1	6854.9	1.96	0.181
Sex	1	2610.8	0.75	0.401
Error (days)	16	3501.4		

preferences among days (day × diet × group), but showed no difference in feeding behavior overall (group between-subject effect, Table 1). Males and females fed similarly (sex between-subject effect, Table 1), both on different days and diets (interaction terms with sex), although there was some evidence of differences in their behavior when feeding on the diets on separate days (day × diet × sex interaction).

There was a strong negative relationship between the change in intake on a given day and the amount ingested the previous day (Figure 1). For example, most larvae decreased food intake on day 2 from day 1, then increased intake on day 3, while larvae were evenly split in their changes on day 4. Examination of the intake plots reveals that for both food and alkaloid intake and on all days (except alkaloid on day 2), larvae that ingested a large quantity ate considerably less the next day, while larvae that ate only a small amount reduced their consumption only slightly. This pattern was the consequence of individuals varying their food and alkaloid intake from day to day (Figure 2) apparently modifying the amplitude of the variation according to at least the previous day's intake. The multiple regression analyses demonstrate that intake of food and alkaloid were related, with both acting as significant and independent predictors of larval feeding behavior (Table 2). The amount of food ingested depended on the amount of food ingested the day before and usually the previous day's alkaloid intake as well. The amount of alkaloid consumed depended on the amount of food eaten the first day and

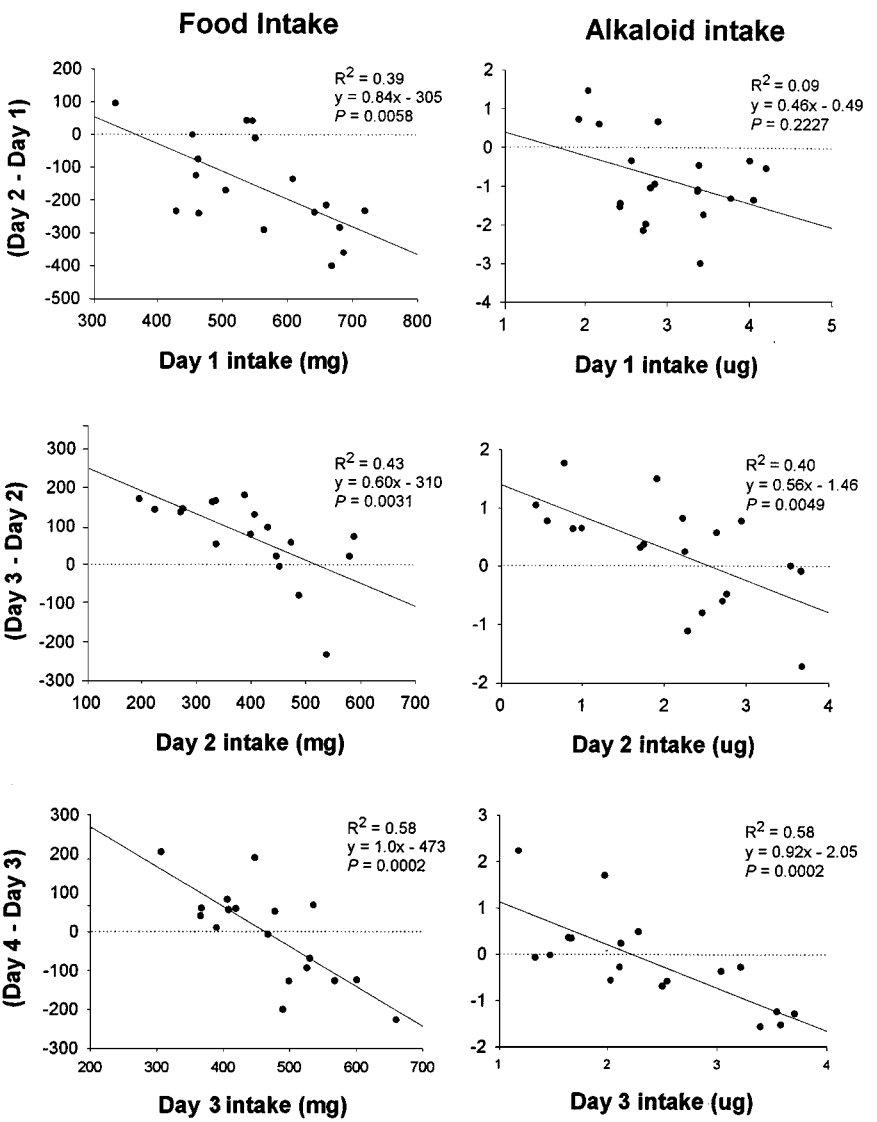


FIG. 1. Regressions of the change in food and alkaloid intake on the amount ingested the previous day. Dotted lines indicate the location of no change in intake from one day to the next.

TABLE 2. MULTIPLE REGRESSION MODELS FOR CHANGE IN FOOD AND ALKALOID INTAKE FROM ONE DAY TO THE NEXT[a]

Dependent variable	*x*	*y*	*z*	Intercept	R^2	*F*	*P*
food: day2-day1	food1	pa1					
	1.30	−115		−202	0.54	8.86	0.003
food: day3-day2	food2						
	0.60			−309	0.43	12.14	0.003
food: day4-day3	food2	food3	pa2				
	−1.02	1.23	92.4	−367	0.78	16.31	<0.001
pa: day2-day1	food1						
	0.004[b]			−1.19	0.15	2.82	0.113
pa: day3-day2	food1	pa2					
	0.004	0.54		−3.24	0.55	9.29	0.002
pa: day4-day3	food1	pa2	pa3				
	0.004	−0.64	1.53	−4.23	0.87	32.48	<0.001

[a] The following abbreviations were used: food = food intake on given day, pa = alkaloid intake on given day. R^2, *F*, and *P* refer to the model overall, not the predictor variables. Predictors left in the model were significant at the 0.15 level.
[b] Not significant at 0.05 level.

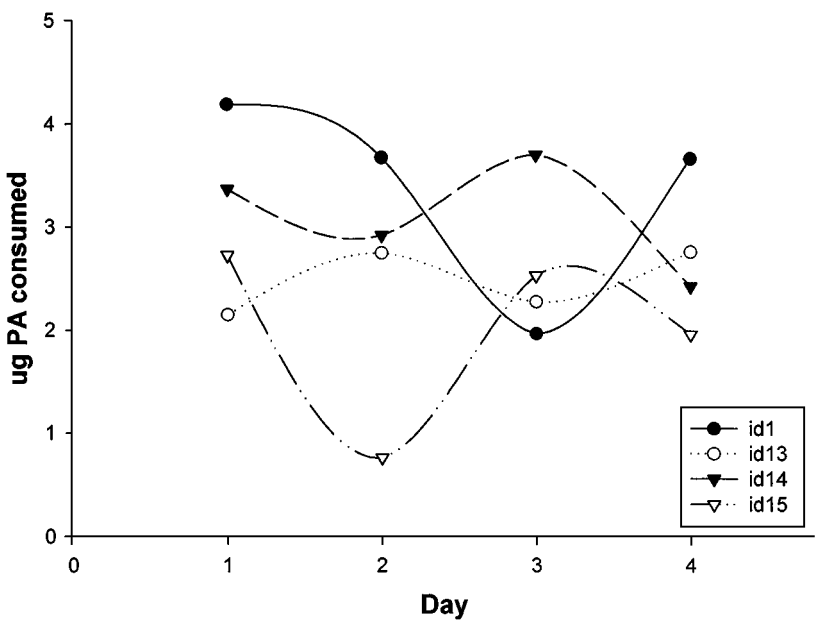


FIG. 2. Alkaloid intake of four representative larvae over the four day trial, showing both the oscillation of intake among days and variation in amplitude of those oscillations.

usually the amount of alkaloid ingested the previous day. Both the simple and multiple regression techniques indicate that the cyclical nature of feeding became more pronounced (stronger negative relationships) and more predictable (higher R^2 values) over time.

Performance Experiments. The first multivariate analysis, using standard performance indices (ECI, ECD, etc.), detected a weak, nonsignificant effect of diet (Wilk's lambda $= 0.434$, $F_{6,24} = 2.14$, $P = 0.086$). The univariate analyses showed the effect of diet was limited to (Figure 3) approximate digestibility (AD) was significantly higher on the seed-powder diet with 0.10% PA, in comparison to either the control (seedless) or 0.41% PA diets. The efficiency of converting ingested food to biomass (ECI) was higher on both seed-powder diets, in comparison to the seedless diet, but not significantly so. The efficiency of converting digested food (ECD) mirrored the response of AD, as would be expected, but the treatments did not differ significantly. The only other notable effect on the performance indices was a weak family effect on AD, suggesting the presence of genetic variation in the ability of larvae to digest a given diet. Neither sex effects, nor family $\times$ diet interactions were detected for the performance indices.

The second multivariate analysis, using measured performance variables, detected a family effect (Wilk's lambda $= 1.79$, $F_{20,57} = 1.79$, $P = 0.045$), but no diet effect. This family effect was found for several variables, including pupal weight (MS $= 6739.1$, $F_{4,21} = 7.38$, $P < 0.001$), total growth (MS $= 3790.8$, $F_{4,21} = 7.38$, $P < 0.001$), growth rate (MS $= 0.884$, $F_{4,21} = 4.95$, $P = 0.006$), and frass production (MS $= 0.252$, $F_{4,21} = 4.76$, $P = 0.007$), but not for duration of the final instar and consumption. Although no effect of diet was detected for the performance variables overall, the univariate analyses showed diet had a weak effect on pupal weight (MS $= 2914.4$, $F_{2,21} = 3.19$, $P = 0.062$), total growth (MS $= 1639.4$, $F_{2,21} = 3.19$, $P = 0.062$), and growth rate (MS $= 0.625$, $F_{2,21} = 3.50$, $P = 0.049$). In particular, larvae reared on the two seed-powder diets grew significantly faster than those reared on seedless diet (Figure 3). This increased growth rate contributed to a shorter duration to pupation for those reared on the diet with the most amount of seeds and occurred in spite of reduced consumption by larvae on the same diet (Figure 3). Weak sex effects were detected only for total growth (MS $= 1552.8$, $F_{1,21} = 3.02$, $P = 0.097$), and final weight (MS $= 2760.5$, $F_{1,21} = 3.02$, $P = 0.097$), with males tending to gain more weight during the final instar and have greater mass at pupation.

Examination of the growth of seven families showed that diet affected pupal weight, weight gain, and growth rate individually and was a main effect in the MANCOVA (Figure 4). Additionally, there were large family and family $\times$ diet effects on all measures of growth (for all $P < 0.001$, data not shown). Some families grew consistently faster than others, while families clearly differed in the diet on which they grew the best.

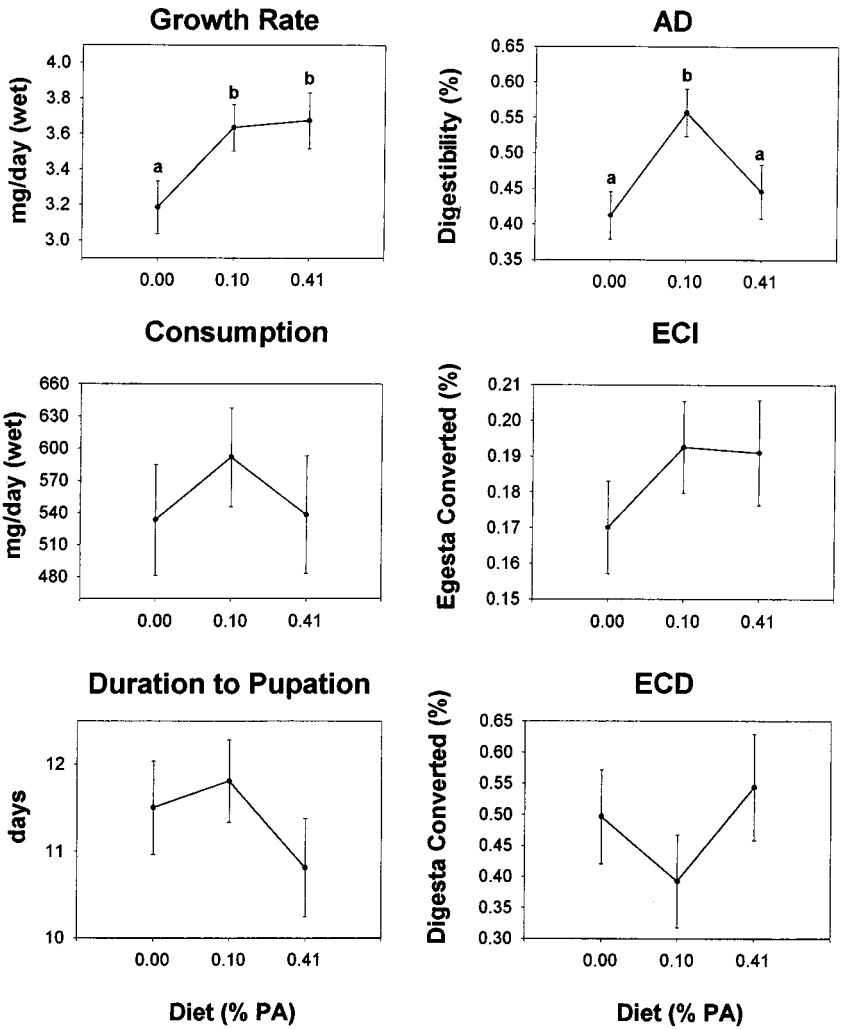
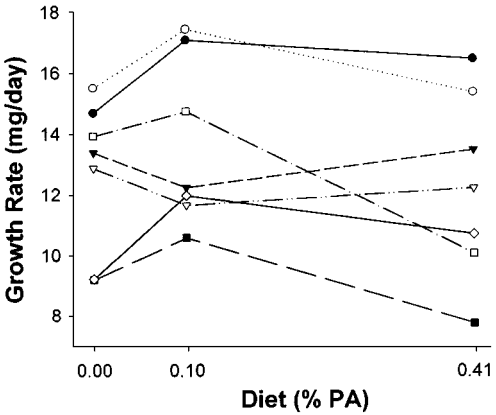


FIG. 3. The responses of three performance variables and the nutritional indices to each of the three diet treatments. Least-squares means and their standard errors are presented. Means with different letters were significantly different. The following definitions were used, after Scriber and Slansky (1981): $ECI = G/F \times 100$, $AD = (F - E)/F \times 100$, $ECD = G/(F - E) \times 100$, $RGR = G/TW$, $RCR = F/TW$ where E = weight of feces, F = weight of food eaten, G = weight gained, T = duration of feeding in days, W = mean weight calculated from initial and final weights. ANCOVA results for AD were: initial weight covariate, MS = 0.019, $F_{1,14} = 2.21$, $P = 0.159$; family effect, MS = 0.025, $F_{4,14} = 2.95$, $P = 0.058$; sex effect, MS = 0.019, $F_{1,14} = 2.24$, $P = 0.156$; diet effect, MS = 0.045, $F_{2,14} = 5.34$, $P = 0.019$; diet × family interaction, MS = 0.012, $F_{8,14} = 1.34$, $P = 0.301$. ACOVA results for growth rate were: initial weight covariate, MS = 0.00067, $F_{1,21} = 0.00$, $P = 0.952$; family effect, MS = 0.884, $F_{4,21} = 4.95$, $P = 0.006$; sex effect, MS = 0.326, $F_{1,21} = 1.83$, $P = 0.191$; diet effect, MS = 0.625, $F_{2,21} = 3.5$, $P = 0.049$; diet × family interaction, MS = 0.240, $F_{8,21} = 1.34$, $P = 0.278$.



Source	df	*P*
Family	12	<0.001
Diet	4	0.051
Diet*Family	24	<0.001
Error	572	

FIG. 4. Norm of reaction plot for growth rate in relation to larval diet. Least-squares means are presented, with a different symbol representing each full-sib family. Results of the multivariate analysis of covariance for measures of growth using initial weight as a covariate are summarized.

DISCUSSION

The diet choice trials demonstrated that *U. ornatrix* larvae actively discriminate among diets with different concentrations of both *Crotalaria* seed powder and monocrotaline. Regardless of the combination offered, larvae in the two-choice trials always ate more of the diet with more PA. However, in the four-choice trials with monocrotaline, larvae consumed more diet with intermediate amounts of PA. These data may be interpreted in two ways. First, larvae may respond more to usaramine than monocrotaline. Second, a low concentration of alkaloid may elicit consistent phagostimulatory behavior, whereas the availability of more alkaloid might elicit more selective behavior. We suspect that the two-choice trials did not test the limits of PA diet choice, considering that the highest concentration of PA in the seed powder diet (0.41%) was less than half that available in the lowest monocrotaline diet (1%). Unfortunately, the constraint of holding nutrient and water content constant in the diet prohibited adding more seed powder, because the total amount of cellulose had been replaced with seed power in the 0.41% diet. That seed powder and monocrotaline are phagostimulants, at least at lower concentrations, is consistent with the observation that pyrrolizidine alkaloids typically act as feeding stimulants in specialists that sequester them (Brown, 1984; Boppré, 1986, 1990; Wink and Schneider, 1990; Bogner and Eisner, 1991).

A Varied Strategy of Dietary Self-Regulation. The four-choice self-selection trial using moncrotaline demonstrated that *U. ornatrix* consumed more diet with

an intermediate amount of alkaloid, but further analyses also revealed a pattern of temporal modulation of dietary alkaloid intake. Individuals varied both their total food and alkaloid intake in an oscillatory manner, with a given larva displaying a consistent amplitude in the oscillation of its consumption. Moreover, the multiple regression analyses clearly showed that changes in alkaloid consumption from one day to the next were dependent on alkaloid intake from previous days, an effect that was distinguishable from and independent of the changes in total food intake. In other words, the results show that the strategy of dietary self-regulation in *U. ornatrix* combines a preference for intermediate alkaloid diets with modulation of the specific amount of alkaloid ingested on a given day.

The observed dependence of feeding behavior on prior intake could be interpreted as a mechanism to obtain a proper balance of nutrients (Rapport, 1980). In particular, the behavior we observed is similar to compensatory feeding in response to dietary deficiencies in protein, carbohydrates, or other essential nutrients (Simpson and Simpson, 1990; Slansky, 1994). Among other insects that sequester for defense, it has not been clear whether there are compensatory responses to ensure that they acquire sufficient quantities from their diet. However, some pyrrolizidine alkaloid specialists actively seek sources of alkaloid (Boppré, 1986; Bogner and Eisner, 1991). We believe our results provide the first demonstration of oscillatory self-regulation of required dietary allelochemical intake in an herbivorous insect. The behavior suggests an active balancing of alkaloid as a nutritive component of the diet.

An alternative interpretation of the observed temporal modulation of alkaloid intake is that the behavior comprises a "dilution strategy" for regulating daily alkaloid intake to avoid autotoxicity (Freeland and Janzen, 1974). If so, then there should be a cost to the ingestion of alkaloids for *U. ornatrix*. Many alkaloids are toxic to a wide range of organisms (Hartmann, 1991) and deterrent to many generalist herbivores (Speiser and Rowell-Rahier, 1991). There certainly are metabolic costs of alkaloid ingestion in some insects (Cresswell et al., 1992), and there may be some cost of PA ingestion for *Creatonotos transiens* (Wink and Schneider, 1988), a PA specialist related to *U. ornatrix*. Based on this evidence, we anticipated finding some decline in the measures of metabolic efficiency, as a result of feeding on diets with alkaloid.

Are There Costs to Alkaloid Intake? Contrary to our expectation, the metabolic efficiency of larvae restricted to diets of low, medium, and high *Crotalaria* seed powder did not differ in a manner that would suggest a cost to ingesting alkaloid. In fact, growth rate and final pupal weight rose as the amount of seed powder in the diet increased. There was no reduction in the efficiency of converting ingested food into biomass (ECI, a combined measure of digestive and metabolic efficiency), or in the efficiency of converted absorbed food into biomass (ECD, metabolic efficiency). There was an increase in approximate digestibility at the intermediate seed powder

concentration and a complementary reduction in ECD at that concentration, but the effect was not linear with respect to concentration and, thus, is difficult to interpret. The only clear concentration-dependent effect of diet was on growth rate. Interestingly, the increase in growth rate on high alkaloid diet was due largely to a shortened development time. Typically, toxic effects reduce growth by extending development time, lowering pupal weight, or both. The pronounced effect on duration indicates a complex trade-off between growth, development time, and pupal mass in *U. ornatrix*.

In spite of the lack of evidence for metabolic costs in the nutritional performance experiment, feeding on a high alkaloid diet did have a negative impact on some genotypes in the population. In the longer-term growth study (three versus one stadia), which comprised a larger sample size of both individuals (318 vs. 48) and families (7 vs. 5), there were large family and small diet effects on growth and pupal weight, with families differing in their performance on the diet presented. Not all families grew faster on the seed powder diets, and most had reduced growth on the highest seed powder diet. These data indicate the presence of genetic variability in the tolerance to dietary alkaloid and are similar to performance and genetic measures of *Junonia coenia* Hubner (Nymphalidae) (Camara, 1997), a butterfly that sequesters iridoid glycosides.

Several caveats are worth mentioning given the lack of evidence for a metabolic cost in the nutritional performance study. First, gravimetric estimations of metabolic costs are sometimes criticized for being insensitive (Schmidt and Reese, 1986; van Loon, 1991). While we agree such assays may not detect a small metabolic cost, we believe the analyses are sufficiently precise to detect a large cost of feeding on alkaloids. Such a large effect would have been consistent with the toxin dilution hypothesis. Second, the concentration of alkaloid in the seed powder diet may not have been high enough or administered over a sufficient duration in the nutritional performance study to detect a metabolic cost. Third, the high quality of the artificial diet, or even the addition of non-PA components of the seed powder, may have offset any negative effects of increasing the PA content of diet. Additional studies that take these limitations into consideration might well detect a small metabolic cost of alkaloid intake in *U. ornatrix*.

We conclude that *U. ornatrix* appears to be well adapted to deal with the moderate levels of alkaloid encountered in its diet. Any negative effects of dietary alkaloid on growth are modest and only apparent after a longer duration of exposure or for some genotypes within the population. The pattern of temporal modulation of intake rate suggests larvae may have fairly sophisticated feeding behaviors that help maximize lifetime accrual of PA, while minimizing the potentially negative effects associated with ingesting too much PA within a short period of time. Because *U. ornatrix* larvae are highly mobile and frequently feed on both high-alkaloid seed pods and low-alkaloid leaf tissues, it is likely that they can detect and modulate

their intake to minimize negative effects. More information about the amount of variation in alkaloid content normally encountered in the field, and the genetic and behavioral responses of *U. ornatrix* to this variation, is needed to better determine whether self-regulation of dietary alkaloids occurs under natural conditions and to what extent it affects components of fitness.

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BIOCHEMICAL RESPONSES OF *Pinus pinaster* TREES TO FIRE-INDUCED TRUNK GIRDLING AND CROWN SCORCH: SECONDARY METABOLITES AND PIGMENTS AS NEEDLE CHEMICAL INDICATORS

M. ALONSO,* M. J. ROZADOS, J. A. VEGA, P. PÉREZ-GOROSTIAGA, P. CUIÑAS, M. T. FONTÚRBEL, and C. FERNÁNDEZ

Xunta de Galicia, Consellería de Medio Ambiente
C.I.T.A. Centro de Investigaciones Forestales y Ambientales, Lourizán
Apdo. 127 36080 Pontevedra, Spain

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Abstract—The biochemical responses to varying levels of localized heat damage to stems and crowns were studied in *Pinus pinaster* trees in Galicia, Spain. The objective was to ascertain the utility of secondary metabolites (total polyphenols, condensed tannins, astringency, free and esterified phenolic acids) and pigments (chlorophylls and carotenoids) as chemical indicators of localized fire damage. The study involved three levels of girdling by trunk heating (0%, 60%, and 75%), three levels of crown scorching (0%, 50%, and 75%), and all combinations of those trunk and crown treatment levels. Secondary metabolites and pigments were analyzed in needles before, during, and up to 8 months after fire treatments. High levels of polyphenols, condensed tannin, and pigments occurred in situations where the sole treatment was 75% crown scorching. Low levels of hydroxycinnamic acids were the result when the treatment was 75% trunk girdling. These responses occurred in the first two months after fire. Thereafter, normal values were observed. Low values of chlorophyll a/b ratio and high levels of free protocatechuic and esterified syringic acids were found to be long-term indicators of trunk girdling damage combined with crown scorching.

Key Words—*Pinus pinaster*, fire effects, needles, secondary metabolites, pigments, indicators.

*To whom correspondence should be addressed. E-mail: marga@inia.es

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INTRODUCTION

Phenolic compounds are a group of secondary products that are almost universally present in higher plants (Harborne, 1980). The phenylpropanoids are a biosynthetic group formed in part via the shikimate pathway. A wide range of environmental stress factors affect this route (Rhodes, 1985). Temporary limitation of photosynthesis due, for example, to short-term drought or temperature extremes, leads to a decrease in carbon gains proportional to the duration of the unfavorable conditions. If these are too severe or prolonged, it takes longer for the plant to reactivate photosynthesis through its repair mechanisms after conditions have improved, if it recovers at all. This situation is referred to as the after-effect of stress (Larcher, 1995).

Fire, as a stress factor, may induce metabolic changes in leaves. Knowing the extent and nature of the metabolic changes in plants is important for the use of prescribed fire. Various effects of fire on trees have been identified. Biophysical parameters may change in forest species affected by direct heat application to the trunk or by prescribed fire, so that it is possible to predict tree survival from these changes (Bará et al., 1990, 1992, 1994).

Leaf composition can be related to flammability. Owens et al. (1998) observed the relationship between susceptibility of *Juniperus* communities to prescribed fires and the composition of volatile oils. Several biochemical changes in trees may occur as a result of fire damage, depending upon the level of crown scorching. These changes affect the relative vigor of damaged trees and appear to increase the attraction of insects and disease to fire damaged trees (Chambers et al., 1986). Studying the effect of a lightning strike on bark beetle susceptibility indicators in loblolly pine, Blanche et al. (1985) found that α-pinene, a primary attractant to beetles, increased by 71% after a lighting strike while the amount of myrcene in oleoresin, a precursor to *Ips* beetle pheromones, increased by 335%. In contrast, they found limonene, which is toxic to bark beetles, decreased, as did the level of β-pinene, a mountain pine beetle repellent. We noted increases in myrcene and α-pinene and decreases in β-pinene in needles of *Pinus pinaster* trees (relative to control trees) six months after they were affected by wildfire (Alonso et al., 1993b). Jakubas et al. (1994) found that secondary metabolite contents (phenolic glycosides, monoterpenes, and resin acids) were lower in burned than in unburned, lodgepole pine bark. Burned bark also contained more crude protein. Low levels of plant secondary metabolites in dead and burned bark may explain why elk and some insects preferentially utilize these trees. High levels of free proline and low levels of hydroxyproline were observed in *Pinus pinaster* trees affected by prescribed fire (Rozados et al., 1992, 1993). Fleck et al. (1996) observed in fire treated *Quercus ilex* that shoot foliage exhibited enhanced photosynthesis, leaf conductance, and Rubisco activity (particularly under high temperatures, irradiance, and vapor pressure deficit conditions) compared with foliage sampled from the lower

canopy in an undisturbed, contiguous stand (control). Higher values of astringency, total polyphenols, condensed tannins, free gallic and *p*-hydroxybenzoic acids, esterified gallic, protocatechuic and syringic acids, and lower free protocatechuic and esterified vanillic acids were observed in needles of *P. pinaster* trees affected by prescribed fire than were found in control trees. In the roots of the same trees, free protocatechuic and *p*-hydroxybenzoic acids were higher, and total polyphenols and esterified protocatechuic acid were lower (Alonso et al., 1998). Significantly more total chlorophyll and carotenoids (per gram of dry leaf tissue) were found in the first leaves that developed from affected trees after a fire than in leaves from unburned oaks (Trabaud and Methy, 1988).

Controlled crown and trunk heat injury treatments of ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) revealed effects of fire on xylem pressure potential, stomatal conductance, and transpiration (Ryan, 1993). Localized heating applied to the trunk of *Pinus halephensis* Mill. determined ecophysiological and morphological responses to fire injury (Ducrey et al., 1996). Rigolot et al. (1994) found that sap flux density and net photosynthesis were the most sensitive indicators of pending tree death. Mortality was nil until girdling exceeded 90% of the circumference. Although partial defoliation reduced stem diameter growth, partial girdling had no effect. The content of free protocatechuic acid was lower and esterified gallic, protocatechuic, and *p*-coumaric were higher than the control in 1-year-old *Pinus pinaster* seedlings after heat application to the trunk (Alonso et al., 1993a).

Many aspects of tree response to fire remain unknown. The aim of this study was to quantify the response of maritime pine (*Pinus pinaster* Ait.) to fire induced crown scorching and trunk girdling and to relate any chemical changes and their timing with the type of fire damage and/or the level of fire injury.

METHODS AND MATERIALS

Experimental Site. The experiment was conducted in an 18-year-old *P. pinaster* stand (dbh 18 cm, height 10.1 m, and density 2500 trees/ha) located in Cotobad (Pontevedra) in the northwest of Spain (700 m elevation, 38% slope). The substrate was granitic, the understory mixed heather (*Calluna vulgaris* and *Erica* spp.) and leguminosae (*Ulex* sp.), the mean annual precipitation of 1800 mm is well distributed throughout the year with a short dry summer season, and mean annual temperature of 10.5°C. The site occurs in clime VI (V) of Allúe's (1990) classification.

Localized Fire Treatments and Sampling. A completely randomized factorial experimental design (45 trees and 5 replicates by treatment) was used to study the effect of localized heat application to *P. pinaster* trees. Three levels of girdling by trunk heating (0%, 60%, 75%), three levels of crown scorching (0%, 50%, 75%), and the combination of both trunk and crown treatments were considered.

A gas flame was used to generate the heat necessary to damage the trunk cambium. The heat reached the trunk through a metal band fastened to the western side of the trunk. The metal band was 16.5 cm wide and 1.5 mm thick, and long enough to cover 60–75% of the perimeter of the tree (appropriate to the assigned treatment), with the lower edge of the band located approximately 35 cm above the forest floor. In the zone where the metal band was to be used, the bark was shaved slightly to reduce its fissures and roughness, thereby achieving a tighter contact between surfaces and a greater uniformity of heat conduction.

Crowns were scorched using a gas flame. The flame was applied to leaves and small branches at a distance of 50 cm for a few minutes until the desired level of healthy crown remained. Crown scorching was always done from below to simulate surface fire action. Leaf surface sheen (caused by melting wax), was used as an indicator of needle death.

All trees were treated between March 4 and 8, 1996. Before application of each treatment, 1-year-old needle samples were collected from the apex of a south-facing fourth whorl branch in four of the five trees treated February 16. We conducted another sampling immediately after treatment on March 12 and additional sampling on March 27, May 9, July 9, and November 6, 1996. Only green needles (not affected directly by heat application) were sampled. Pigment concentrations from fresh needles and other biochemical parameters were quantified from freeze-dried needles.

Analytical Methodology. Needle astringency was measured with hemoglobin derived from fresh bovine blood as a binding substrate in protein precipitation of tannins according to the method of Schultz and Baldwin (1981). Condensed tannins were measured with H_2SO_4/vanillin reagent (Scalbert et al., 1989), and total polyphenols with Folin Ciocalteau reagent (Dominguez, 1987). All of these determinations were made with methanol–water extracts (50:50 v/v), with the addition of $KHSO_3$ to prevent oxidation of phenolic compounds during the extraction (Peng and Jay-Allemand, 1991). Free and esterified phenolic acids (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, syringic, *p*-coumaric and ferulic) were analyzed by HPLC in 0.1% $KHSO_3$ water extract (Alonso et al., 1993a, 1998).

Pigments were extracted in dimethyl sulfoxide at 60°C during 8 hr immediately after the samples arrived at the laboratory (Hiscox and Israelstam, 1979). Spectrophotometrical readings were made in filtered solutions at 663, 646 and 470 nm (Lichtenthaler and Wellburn, 1983).

Statistical Analysis. Analysis of variance was undertaken for one between factor (treatment), including nine levels, and one within factor (sampling date) with one preburn and five postburn levels using a probability level of $P \leq 0.05$. Preplanned comparisons were made to evaluate differences between treatments by sampling dates throughout the period of study. Comparison of treatments included the effect of crown scorching only, crown scorching with 60% girdling by trunk heating, crown scorching with 75% girdling by trunk

heating only, girdling by trunk heating with 50% crown scorching, girdling by trunk heating with 75% crown scorching, and simultaneous and similar damages to trunk and crown.

RESULTS AND DISCUSSION

Fire Damage to Crowns

Crown damage resulted in increases in needle polyphenols (Figure 1A) and condensed tannins (Figure 1B). This effect was observed two months after the fire in all trees subjected to crown scorching. Trees with 75% crown scorching had higher polyphenol and condensed tannin levels than control trees for the postfire treatment dates. Trees damaged only by crown scorching at a 75% level exhibited an increase in free gallic acid 20 days after treatment (Figure 1C), and this effect could still be detected two months later. Trees with 75% crown scorching showed higher levels than control trees.

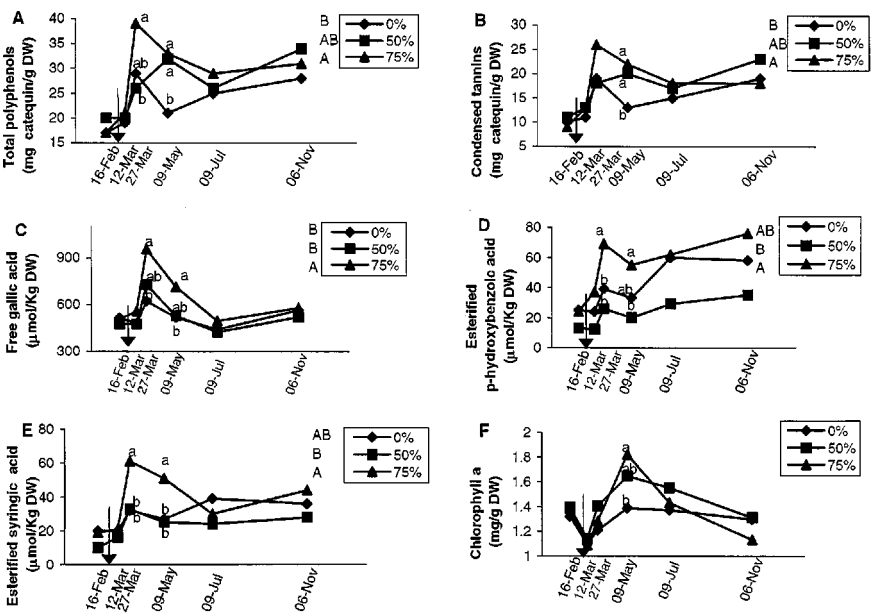


FIG. 1. Effects of different percentages of crown scorching on needle total polyphenols (A), condensed tannins (B), free gallic acid (C), esterified *p*-hydroxybenzoic acid (D), esterified syringic (E), and chlorophyll *a* (F). The vertical arrow refers to fire treatment date (March 4–8). Different lowercase show significant differences between fire treatments on each sampling date ($P \leq 0.05$). Different capital letters indicate significant differences between treatments, considering the set of data after fire treatment ($P \leq 0.05$).

Twenty days after treatment, a high level of esterified *p*-hydroxybenzoic acid was observed in trees with 75% crown scorching compared to the levels in control trees (Figure 1D). Esterified syringic acid increased 20 days after treatment with 75% crown scorching and continued to rise until analysis two months later (Figure 1E). Thereafter, the level appeared to stabilize. Two months after fire treatment, the chlorophyll *a* content was significantly higher in trees with 75% crown scorching than in control trees, while trees with 50% of the crown affected had values intermediate between control and trees with 75% crown affected (Figure 1F). This effect disappeared in later samplings. The same result was obtained for chlorophyll *b*, total chlorophyll, and carotenoids (data not shown). In a previous study conducted on the same species two months after a mild intensity prescribed fire, high levels of polyphenols, tannins, and free gallic acid were noted in comparison to control trees (Alonso et al., 1998). In that study, the prescribed fire also affected a high percent of crown volume.

It can be concluded that 50% crown scorching by fire in *P. pinaster* trees does not modify the level of metabolites found in green needles. However, when 75% crown scorching occurs, increases in needle metabolite levels can be seen two months after treatment. Changes in phenolic acid levels occur 20 days after treatment. This suggests that secondary metabolism is activated to produce defense compounds, such as polyphenols, that might play a defensive role (Harborne, 1994; Hammerschmidt and Schultz, 1996) and may be enhanced as a consequence of crown scorch. Later, primary metabolism is enhanced to restore the photosynthetic activity that was diminished by crown scorching. Decreases in photosynthesis have been correlated with decreases in chlorophyll content by the action of phenolic compounds (Rama et al., 1997). These transitory effects indicate that irreversible changes did not occur, since carbon balance seemed to be restored four months after crown fire treatment. Total polyphenols, condensed tannins, and pigments may be good chemical indicators of crown damage alone. Furthermore, it is important to point out that severe crown scorching produces only temporary changes in these biochemical parameters. Botelho et al. (1998) suggested that relatively intense prescribed burns that result in severe crown scorching had a temporary and negligible impact upon growth rate (diameter at breast height and total height). Defoliation has little effect on survival until almost all of the foliage is removed in pine: young vigorous pines can survive fire-caused defoliation of more than 80% (Rigolot et al., 1994).

Fire Damage to Trunks

Twenty days after the fire treatment to the trunk, there was an increase in esterified gallic acid in trees with 75% trunk girdling (Figure 2A). Damage to only the trunk caused decreases in esterified *p*-coumaric acid in the trees with 75% trunk girdling two months after treatment (Figure 2B). For the same trees,

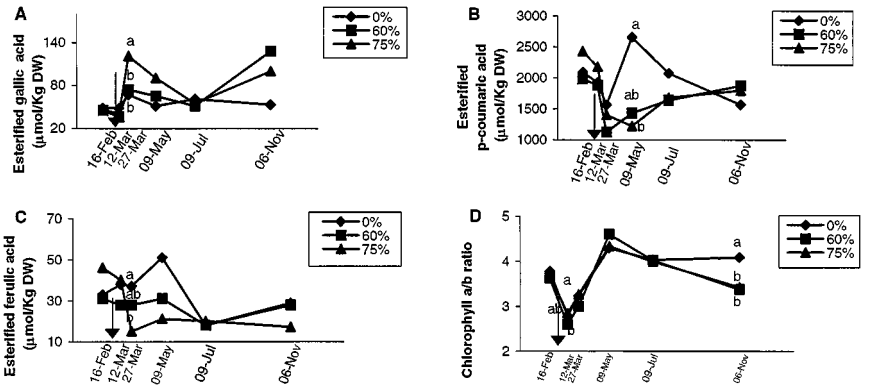


FIG. 2. Effects of different percentages of trunk girdling on needle esterified gallic acid (A), esterified *p*-coumaric (B), esterified ferulic acid (C), and chlorophyll *a*/*b* ratio (D). The vertical arrow makes reference to fire treatment date (March 4–8). Different lowercase show significant differences between fire treatments on each sampling date ($P \leq 0.05$). Different capital letters indicate significant differences between treatments, considering the set of data after fire treatment ($P \leq 0.05$).

esterified ferulic acid decreased 20 days after treatment (Figure 2C). Ferulic acid is a precursor of coniferyl alcohol, the principal component of lignin in gymnosperms (Haslam, 1993). Fire injury to trunks may induce an increment in lignin production to repair damaged tree tissues, by utilization of ferulic acid and reducing its esterification.

In the final sampling, eight months after treatment, chlorophyll *a*/*b* ratios showed significant decreases for both fire treatments compared to control trees (Figure 2D). A 60% trunk girdling in *P. pinaster* trees was not sufficient to change metabolite levels in green needles, but 75% trunk girdling induced a specific decrease in hydroxycinnamic acids two months after the treatment. The chlorophyll *a*/*b* ratio may be useful for revealing long-term effects in treatment levels applied to tree trunks.

In another study, 1-year-old *P. pinaster* seedlings exposed twice to trunk heating showed decreases in electrical conductivity only in the damaged zone of the trunk (Bará et al., 1990). In the same seedlings, an increase in esterified gallic acid was observed four months after the second treatment, while the levels of esterified *p*-coumaric and ferulic were higher than those in control trees (Alonso et al., 1993a). However, in this study, esterified *p*-coumaric and ferulic acids levels were lower than those in control trees when trunk girdling alone was applied, suggesting different metabolic responses in young and mature trees. Vega et al. (1998), in a *P. pinaster* stand treated with prescribed burning, found that bark showed a high capability to effectively protect cambium for diameters ranging

from 9 to 40 cm. In these situations, temperature levels measured in the cambium, 25 cm above ground, were much lower than those registered on the outer bark surface (28.6°C instead of 662.9°C). This can explain why few changes were observed in severe trunk-affected trees. In a similar experiment where localized heating was applied to the trunks of young *P. halepensis*, Ducrey et al. (1996), found that the threshold for destruction of the cambium, in absence of any damage to foliage, occurred when 80–100% of the circumference of the base of the trunk was destroyed. They also observed that the behavior of partially burned pines was not significantly different from controls for all physiological variables studied (sap flux density, micro variations in trunk diameter, electrical resistance, photosynthesis, and stomatal conductance).

Combined Fire Damage to Trunks and Crowns

Trees with 50% Crown Scorching, Additional Trunk Girdling. In the most affected trees, a high level of astringency appeared immediately after fire. Fifteen days later the same results appeared in all trunk-damaged trees. In general, trees with 75% trunk girdling showed higher values than trees with 0% trunk girdling (Figure 3A). In the same trees, free protocatechuic acid, the most abundant phenolic acid in *P. pinaster* needles (Alonso and Bará, 1989), increased with 75% trunk girdling two months after fire treatment. This difference remained for eight months after fire treatment. In general, all trees with trunk damage showed higher values than trees with 0% trunk girdling in postfire dates (Figure 3B).

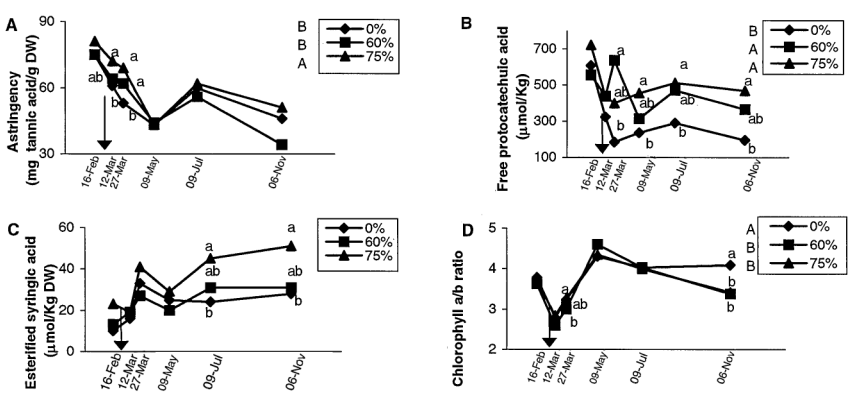


FIG. 3. Effect of different percentages of trunk girdling in trees with 50% crown scorching on needle astringency (A), free protocatechuic acid (B), esterified syringic (C), and chlorophyll *a*/*b* ratio (D). The vertical arrow makes reference to fire treatment date (March 4–8). Different lowercase letters show significant differences between fire treatments on each sampling date ($P \leq 0.05$). Different capital letters indicate significant differences between treatments, considering the set of data after fire treatments ($P \leq 0.05$).

Esterified syringic acid level in trees with 75% trunk girdling was higher than in trees with 0% trunk girdling. This increase was detected four months after fire treatment and persisted to the end of the experiment (Figure 3C). Trees with 60% trunk girdling showed lower chlorophyll *a*/*b* ratios than trees with 0% trunk girdling 20 days after fire treatment. In November, all trees with trunk girdling showed lower values than trees with 0% trunk girdling. In general, all trunk-girdled trees had lower values than trees with 0% trunk girdling at post-fire treatment dates (Figure 3D).

Trees with 75% Crown Scorching, Additional Trunk Girdling. In cases where trees were subjected to 75% crown scorching combined with 75% trunk girdling, lower values in needle astringency than those in trees with 0% trunk girdling were observed for postfire data (Figure 4A). A decrease in esterified syringic acid for all trees with damage to the trunk 20 days and two months after fire treatments was noted and, in general, in postfire data in comparison to trees with 0% trunk girdling (Figure 4B). This is an inverse effect from that seen in trees with 50% crown scorching and additional 75% trunk girdling (Figure 3A,C).

Two months after fire treatment, levels of chlorophyll *b* decreased in trees with 60% trunk girdling relative to trees with 0% trunk girdling. In the final sampling, trees with 75% trunk girdling showed higher values than those with 0% trunk girdling (Figure 4C). All the trees with trunk girdling showed lower values for chlorophyll *a*/*b* ratios than trees with 0% trunk girdling in the final sampling (Figure 4D).

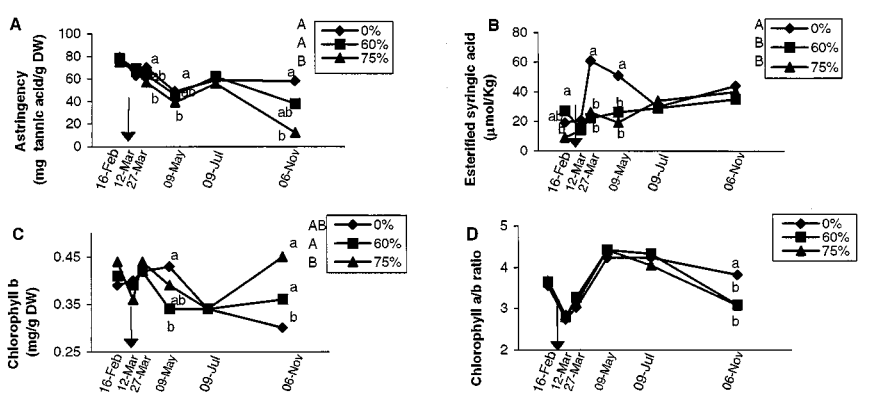


FIG. 4. Effect of different percentages of trunk girdling in trees with 75% crown scorching on needle astringency (A), esterified syringic acid (B), chlorophyll *b* (C), and chlorophyll *a*/*b* ratio (D). The vertical arrow refers to the fire treatment date (March 4–8). Different lowercase letters show significant differences between fire treatments on each sampling date ($P \leq 0.05$). Different capital letters indicate significant differences between treatments, considering the set of data after fire treatment ($P \leq 0.05$).

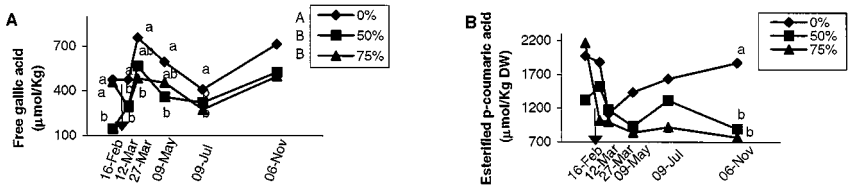


FIG. 5. Effect of different percentages of crown scorching in trees with 60% trunk girdling on needle free gallic acid (A) and esterified *p*-coumaric acid (B). The vertical arrow refers to fire treatment date (March 4–8). Different lowercase letters show significant differences between fire treatments on each sampling date ($P \leq 0.05$). Different capital letters indicate significant differences between treatments, considering the set of data after fore treatment ($P \leq 0.05$).

Trees with 60% Trunk Girdling, Additional Crown Scorching. In these trees, girdling in combination with 75% crown scorching produced a decrease of free gallic acid immediately after fire treatment, with the effect observable for four months. The trees showed lower free gallic acid values in needles than trees with 0% crown scorching (Figure 5A). The same trees contained less esterified *p*-coumaric acid in needles than the trees with 0% crown scorching in the final sampling (Figure 5B).

Trees with 75% Trunk Girdling, Additional Crown Scorching. We observed an increase in free vanillic acid in trees with 75% crown scorching two and four months postfire compared to trees with 0% crown scorching. In general, trees with 75% crown scorching showed higher free vanillic acid content than the trees with 0% crown scorching (Figure 6).

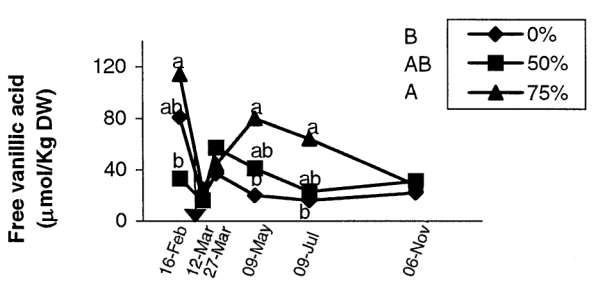


FIG. 6. Effect of different percentages of crown scorching on trees with 75% trunk girdling on needle free vanillic acid. The vertical arrow refers to fire treatment date (March 4–8). Different lowercase letters show significant differences between fire treatments on each sampling date ($P \leq 0.05$). Different capital letters indicate significant differences between treatments, considering the set of data after fire treatment ($P \leq 0.05$).

In combined fire damage to trunk and crown, we observed characteristic needle astringency and esterified syringic acid changes. When additional trunk damage was applied to low-level crown damaged trees, these parameters increased, and the same parameters decreased if trunk damage was added to the trees with 75% crown scorching. This may indicate that, when damage exceeds a determined level, the tree does not react to activate the production of defense compounds and their levels diminish. Beyond eight months after fire treatment, the chlorophyll *a*/*b* ratio dropped, indicating a decrease in the efficiency of the conversion to energy and suggesting the presence of shadelike chloroplast in the needles. This finding is common to all girdled trees, with or without crown scorch, but is not observed in trees that are only crown scorched, suggesting a direct relationship between trunk girdling and long-term changes in the photosynthetic apparatus.

Simultaneous and Similar Damage to Trunks and Crowns

Twenty days after fire treatments, we noted high levels of esterified gallic acid in the most affected trees (75% trunk girdling and 75% crown scorching) compared to control trees. In July, this effect was observed in all affected trees. The mean of the postfire data in most affected trees was higher than that found in control trees (Figure 7A). Chlorophyll *a* content in mildly affected trees (60% trunk girdling and 50% crown scorching) showed higher values than control trees 20 days after fire treatment. Two months after fire, all treated trees showed higher chlorophyll *a* content than control trees (Figure 7B). Similar results were obtained for total chlorophyll.

In conclusion, polyphenols, condensed tannins, and pigments may reflect crown scorching damage alone, while hydroxycinnamic acids can be useful indicators of trunk damage. The chlorophyll *a*/*b* ratio seems to be a long-term

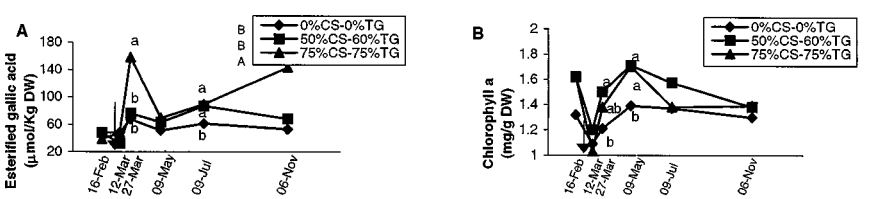


FIG. 7. Effects of different percentages of simultaneous and similar damages to trunk and crown on needle esterified gallic acid (A), and chlorophyll *a* (B). The vertical arrow refers to fire treatment date (March 4–8). CS: crown scorch, TG: trunk girdling. Different lowercase letters show significant differences between fire treatments in each sampling date ($P \leq 0.05$). Different capital letters indicate significant differences between treatments, considering the set of data after fire treatment ($P \leq 0.05$).

indicator of trunk damage, both alone or combined with crown scorching. Esterified syringic acid variations can reflect crown scorching, but this changes with additional trunk heating levels. Metabolic changes produced by both crown scorching and trunk heating show up in the first two months after fire. Thereafter, values return to normal. When both trunk and crown are affected by fire, some metabolites show variations that are detectable four and even eight months after fire. Under the conditions of this study, irreversible physiological disruption was not detected eight months after treatment, showing the potential use of mild intensity prescribed burns for *Pinus pinaster.* Nevertheless, the timing of fire treatment can also be important. Defoliation in summer can be particularly damaging because this is a time of rapid assimilative storage, while in winter the carbohydrate reserves are greater. Therefore, the conclusions of this study are limited to winter fires and any extrapolation to fire treatments carried out in summer must be avoided.

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DISTRIBUTION OF LOCOWEED TOXIN SWAINSONINE IN POPULATIONS OF *Oxytropis lambertii*

M. H. RALPHS,[1,*] S. L. WELSH,[2] and D. R. GARDNER[1]

[1]*USDA/ARS Poisonous Plant Laboratory*
1150 E. 1400 N., Logan, Utah 84341

[2]*Bean Natural History Museum*
Brigham Young University
Provo, Utah 84602

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Abstract—*Oxytropis lambertii* has been considered to be one of the major locoweeds responsible for livestock poisoning on rangelands, but there has been much confusion as to its taxonomic identity. The objective of this study was to conduct a field survey of several populations of each of the three varieties [var. *lambertii* Pursh; var *bigelovii* A. Gray; var. *articulata* (E. Greene) Barneby] to document the presence or absence of the locoweed toxin, swainsonine. Swainsonine was found at detectable levels (>0.001% dry weight) in only five populations of var. *bigelovii* in the southwest portion of its distribution in southern Utah, Arizona, and southwestern New Mexico, USA. No swainsonine was detected in populations in the northeast areas of its distribution (eastern Utah, Colorado, northeastern New Mexico, USA). The other varieties, *articulata* and *lambertii*, also did not contain swainsonine. It is suspected that a plant fungal endophyte may be responsible for the high variability in swainsonine content in populations of *O. lambertii*.

Key Words—Lambert locoweed, crazyweed, *Oxytropis lambertii*, swainsonine, livestock poisoning, toxic plant, poisonous plant.

INTRODUCTION

Locoweeds (*Astragalus* and *Oxytropis* spp.) cause widespread poisoning of livestock in the western United States (Kingsbury, 1964). Much of the early toxicological research was conducted on the complex of species then called *Aragallus lambertii* (Marsh, 1909), which included both the white-flowered and the lavender-flowered groups of plants. Later Marsh (1919) referred to the white locoweed as

* To whom correspondence should be addressed. E-mail: mralphs@cc.usu.edu

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Astragalus lambertii. Butcher et al. (1953) were first to point out that the plant with which Marsh conducted most of his early toxicology research was indeed the white flowered *O. sericea*. Taxonomists have separated the two species based on the color of their flowers (*O. sericea* is white to slightly yellow; *O. lambertii* is purple violet or lavender) and the dolabriform (ox head-like) pubescence that is unique to *O. lambertii* (Barneby, 1952; Welsh, 2001).

The confusion in taxonomy complicates the question as to whether *O. lambertii* is really a locoweed and whether it has been responsible for poisoning livestock. Literature reviews of the locoweeds all include *O. lambertii* as one of the major locoweeds (Marsh, 1919; Kingsbury, 1964; James et al., 1981; Ralphs and James, 1999). However, there is no empirical evidence of poisoning attributable to *O. lambertii*. The objective of this research was to document the presence of the locoweed toxin swainsonine in *O. lambertii*.

METHODS AND MATERIALS

A literature review was conducted of the toxicology and chemistry literature for any reference to swainsonine in *O. lambertii*. A widespread field survey of *O. lambertii* populations was conducted and laboratory analysis performed to determine the presence or absence of swainsonine. Voucher specimens are held in the S. L. Welsh Herbarium in the Bean Natural History Museum, Brigham Young University, Provo, Utah.

Known populations of each of three varieties representing the entire geographical distribution of that variety were sampled in June 2000 (Table 1). Ten individual plants were collected at each site. Half of each plant was pressed for identification. The other half was air dried in a forced air oven for 48 hr at 60°C and then ground through a cyclone grinder. Samples were analyzed for the locoweed toxin swainsonine by the procedures described by Gardner et al. (2001). Detection limit of swainsonine was 0.001% of dry weight. Swainsonine concentration was compared by one-way analysis of variance to determine differences between locations.

RESULTS AND DISCUSSION

There are few reports in the literature of *O. lambertii* containing swainsonine (Table 2). Molyneux et al. (1989, 1991) reported the presence of swainsonine in *O. lambertii* by using a thin-layer chromotography technique. Later samples from the Henry Mountains in southeast Utah, and from Livermore, Colorado, showed swainsonine levels of 0.031 and 0.007%, respectively (Molyneux, unpublished data). Fox et al. (1998) reported *O. lambertii* from western and northern New Mexico contained swainsonine. Braun (1999) also reported swainsonine in

TABLE 1. VARIETIES OF *Oxytropis lambertii* VAR. *bigelovii*, STAGE OF GROWTH AND SAMPLING SITES

Variety/ voucher	Stage	Location	GPS	Soil/Site	Plant community
bigelovii					
27661[a]	Seed	Kanab, UT	37°06′19″N 111°51′28″W	Sandy hills	Desert shrub
27665[a]	Veg	Flagstaff, AZ	37°06′41″N 111°51′28″W	Sandy loam foothills	Ponderosa pine openings
27667[a]	Veg	Springerville, AZ	34°00′49″N 111°10′48″W	Gravely hills	Pinyon/juniper woodland
27668[a]	Veg	Kingston, NM	32°52′51″N 107°51′55″W	Rocky, mountain slopes	Ponderosa pine/alligator juniper forest
27669[a]	Bud	Winston, NM	33°21′43″N 107°34′41″W	lóamy valley	Blue grama grassland
27672	Flo	Ocate, NM	36°15′11″N 105°02′32″W	Gravely hills	Ponderosa pine
440981	Flo	Capulin, NM	30°41′25″N 104°08′35″W	Rocky loam sidehill	Shortgrass prairie
440982	Flo	Sophia, NM	36°28′06″N 103°59′54″W	Lóamy flats	Shortgrass prairie
440980	Flo	Ft. Collins, CO	40°56′39″N 105°15′33″W	Gravely foothills	Mixed grass prairie
440983	Flo	Ferron, UT	39°06′57″N 111°17′36″W	Sandy mountain bench	Low sage, bluebunch wheatgrass
articulata					
27689	Pod	Knowles, OK	36°55′15″N 100°18′23″W	Rolling hills	Tall grass prairie
27697	Pod	Buffalo, OK	36°48′44″N 99°46′22″W	Rolling hills	Tall grass prairie
27698	Pod	Meade, KA	37°10′09″N 100°23′03″W	Calcareous breaks	Tall grass prairie
lambertii					
27704	Flo	Sidney, NB	41°09′18″N 103°05′18″W	Limestone bluff	Mixed grass prairie
27717	Flo	Hot Spring, SD	43°24′35″N 103°20′23″W	Gravely hills	Ponderosa pine, mixed grass prairie
27721	Flo	Lusk, WY	43° 05′12″N 104°19′36″W	Sandy/gravel hills	Mixed grass prairies

[a] Populations containing swainsonine.

O. lambertii in Grant County, New Mexico, that was associated with a fungal endophyte.

A recent grazing study comparing the relative palatability of *O. lambertii* with *O. sericea* growing together on short-grass prairie in north-central Colorado, showed that swainsonine was not present in detectable amounts in samples of

TABLE 2. REPORTED ACCOUNTS OF *O. lambertii* CONTAINING SWAINSONINE

Date	Location	Reference	Swainsonine (%)
1986	Henry Mt., Wayne Co., UT	Molyneux (unpubl.)	0.031
n.d.	Livermore, Larimer Co., CO	Molyneux (unpubl.)	0.007
1989	no location given	Molyneux et al. (1991)	>0.01[a]
1987	Winston, Sierra Co., NM	Fox et al. (1998)	0.099
1991	Winston, Sierra Co., NM	Fox et al. (1998)	0.021
1991	Ocate, Moro Co., NM	Fox et al. (1998)	0.026
1999	Grant Co., NM	Braun (1999)	0.576
1999	Colfax Co., NM	Braun (1999)	0.0006

[a] Presence detected by thin-layer chromotography.

O. lambertii taken from different pastures in 1998 and 1999 (Ralphs et al., 2001). Samples of *O. lambertii* collected from another research location in northeastern New Mexico also did not contain swainsonine (Ralphs, unpublished data).

There are three varieties of *O. lambertii*. Variety *bigelovii* A. Gray is separated from the other two by having stipitate pods (slender stalklike base). It occurs from southeastern Wyoming, southward through the foothills and mountains of Colorado and New Mexico, and westward into eastern Utah and Arizona. Variety *lambertii* Pursh is separated from var. *articulata* (E. Greene) Barneby by its longer calyx teeth, about one third as long as the tube and shorter flowers, <18 mm. It occurs on prairies from North Dakota to Kansas with a few populations in eastern Wyoming. Variety *articulata* has short calyx teeth, about one fifth as long as the tube, and flowers 18–25 mm long. It occurs in southwestern Kansas, western Oklahoma, the Texas panhandle, and northeastern New Mexico (Welsh, 2001).

Variety *bigelovii* is the most widespread of the three varieties. Ten populations were sampled in Colorado, Utah, Arizona, and New Mexico (Table 1). Three populations of var. *articulata* were sampled in Oklahoma and Kansas. Three populations of var. *lambertii* were sampled in Wyoming, South Dakota, and Nebraska.

The locoweed toxin is a trihydroxy indolizidine alkaloid, swainsonine (Figure 1) (Molyneux and James, 1982). Detectable levels of swainsonine were found in only five of the 10 populations of *bigelovii* located in the southwestern geographical areas of its range (Table 3). The other five populations in its eastern range did not contain detectable levels. The other two varieties also did not contain detectable levels of swainsonine.

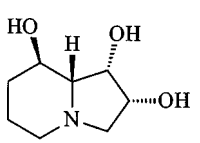


FIG. 1. The toxic alkaloid swainsonine found in locoweeds.

TABLE 3. LOCATIONS IN 2000 SURVEY WHERE *O. lambertii* CONTAINED SWAINSONINE (MEAN ± SE)

Location	Mean (%)	Range (%)
Flagstaff, AZ	0.054 ± 0.027	0.022–0.106
Winston, NM	0.038 ± 0.035	0–0.068
Springerville, AZ	0.026 ± 0.021	0–0.065
Kingston, NM	0.016 ± 0.013	0–0.043
Kanab, UT	0.008 ± 0.016	0–0.047

Four of the five southwestern populations of *bigelovii* containing swainsonine were in the vegetative or bud stage of growth when they were harvested in June (Table 1), indicating they flowered later in the summer. The Kanab, Utah, population that contained swainsonine had flowered early in the spring. The remainder of the *bigelovii* populations and the other two varieties were in the flower and pod stages when harvested, indicating they were spring-flowering plants. The summer monsoonal precipitation pattern, in which the majority of the annual precipitation occurs as summer thunder storms, is responsible for the summer-flowering populations. Although summer thunderstorms are common in areas of the other varieties and populations, there is sufficient winter and spring precipitation to support spring-flowering populations. The presence of swainsonine in both spring- and summer-flowering plants suggests precipitation patterns and subsequent flowering times do not influence presence of swainsonine.

Of the *bigelovii* populations containing swainsonine, the Flagstaff, Arizona, summer-flowering samples had the highest concentrations, while the spring-flowering samples at Kanab, Utah (100 miles away) had the lowest concentrations. Intermediate concentrations occurred in samples from Springerville, Arizona, and Kingston and Winston, New Mexico. Not all of the plants collected at these five locations contained swainsonine. Flagstaff was the only location in which all plants contained it. At Kanab, Utah, only half of the plants contained detectable levels of swainsonine. The range in concentration at each location was fairly large (Table 3).

There is a discrepancy between the presence of swainsonine in previous studies and our results. Fox et al. (1998) reported *O. lambertii* at Ocate, New Mexico, contained swainsonine, while our samples from Ocate did not contain swainsonine at detectable levels. Molyneux (unpublished data) found swainsonine in a sample from Livermore, Colorado, which is near our Ft. Collins, Colorado, population that did not contain swainsonine. Braun's (1999) sample from Colfax County, New Mexico, contained very small amounts of swainsonine (0.0006%), while our Colfax County sample was below the detectable limit (<0.001%).

It appears that the presence of swainsonine in *O. lambertii* is not genetic. Varieties *articulata* and *lambertii*, and half of var. *bigelovii* did not contain detectable

levels. Furthermore, there were individual plants within most collection sites that did not contain swainsonine, ruling out environmental influences.

It has been suggested that swainsonine in locoweeds may be produced by endophytes. Swainsonine is also produced by the fungus *Rhizoctonia leguminicola* in moldy forages (Broquist et al., 1984) and the fungus *Metarhizium anisopliae* (Hino et al., 1985). Braun (1999) found a fungal endophyte tentatively identified as *Alternaria nova* in toxic locoweed species, and reported a near perfect association between the presence of the endophyte and swainsonine in locoweed species. Recent research suggests that the fungus *Rhizoctonia leguminicola* was misclassified and should be in the phylum Ascomycota, which also contains *Alternaria nova* (McGinn et al., 1999). Analysis of our samples for the fungal endophyte showed a strong correlation ($r = 0.92$) with the presence of swainsonine (Gardner et al., 2002). The endophyte infection was limited to the five populations of variety *bigelovii* that contained significant levels of swainsonine, but there were a few plants infested by the endophyte that did not contain swainsonine and a few plants containing swainsonine in which the endophyte was not detected. Further research is needed to determine if swainsonine in locoweed is produced by an endophyte or if the stress from the endophyte augments swainsonine synthesis. This may explain why some populations or individual plants within a population do not contain it.

It is curious to note that the populations of *O. lambertii* var. *bigelovii* at Ft. Collins, Colorado, and at Capulin, Sophia, and Ocate, New Mexico, that did not contain swainsonine are growing together with *O. sericea*, which does contain fairly high levels (0.04–0.11) (Ralphs et al., 1993, 2001). The opportunity for infection by endophytes between these species would seem possible.

In summary, some populations of *O. lambertii* var. *bigelovii* in the southwest areas of its distribution contained swainsonine (Kanab, Utah; Flagstaff and Springerville, Arizona; Kingston and Winston, New Mexico). However, populations in the northeastern areas of its distribution did not contain detectable levels. Populations of the other two varieties, *O. lambertii* var. *articulata* and *lambertii*, also did not contain detectable levels of swainsonine. However, previous research showed some of the northern populations did. There is some evidence that swainsonine is produced by an endophyte. It is likely that other populations of *O. lambertii* may contain the endophyte and produce swainsonine. Further research will be conducted to confirm the presence of endophytes and their contribution to swainsonine.

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RESPONSES TO MAJOR CATEGORIES OF FOOD CHEMICALS BY THE LIZARD *Podarcis lilfordi*

WILLIAM E. COOPER, JR.,[1,*] VALENTÍN PÉREZ-MELLADO,[2] and LAURIE J. VITT[3]

[1]*Department of Biology*
Indiana University-Purdue University at Fort Wayne
Fort Wayne, Indiana 46805

[2]*Departamento de Biologia Animal*
Universidad de Salamanca
37071 Salamanca, Spain

[3]*Sam Noble Oklahoma Museum of Natural History*
and Department of Zoology
University of Oklahoma
2401 Chataqua Avenue
Norman, Oklahoma 73072-7029

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Abstract—Many lizards are capable of identifying food using only chemical cues from food surfaces, but almost nothing is known about the types of compounds that are effective stimuli. We experimentally studied lingual and biting responses by a lacertid lizard, *Podarcis lilfordi*, to single representatives of three major categories of food chemicals, sucrose as a carbohydrate, pure pork fat as a mixture of lipids, and bovine gamma globulin as a protein. In 60-sec trials in which stimuli were presented on cotton swabs, the lizards detected all three stimuli, exhibiting more tongue-flicks, licks, or bites, or a greater tongue-flick attack score (TFAS; overall measure of response strength to prey stimuli) than to deionized water. The initial response to all stimuli was tongue-flicking, but the lizards discriminated among the types of chemical stimuli. After preliminary tongue-flicks, the lizards responded to sucrose solutions by licking at high rates, to pure pork fat by biting, and to protein by a combination of additional tongue-flicks and biting. Biting is a feeding response to prey or solid plant material. Licking is a feeding response to sugars in nectar or ripe fruit. Its frequency increased with sucrose concentration. Our data suggest that lizards can identify several types of chemicals associated with food and direct feeding attempts to sources of such chemicals in the absence of visual cues.

*To whom correspondence should be addressed. E-mail: cooperw@ipfw.edu

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INTRODUCTION

Food flavors, based on a combination of olfactory and gustatory responses to chemical properties of food, are extremely important to evaluations of food by humans. Many other animals are able to locate and evaluate foods on the basis of perceptual qualities associated with classes of compounds, such as the sweetness of sugars and the bitterness of alkaloids. For many snakes and lizards, for which flavors might be determined jointly by vomerolfaction, olfaction, and gustation, very little is known about the importance of particular categories of food chemicals in stimulating chemosensory investigation and feeding responses.

Lizards that are active foragers (Cooper, 1995, 1997, 2000a) or include substantial quantities of plants in the diet (Cooper, 2000b,c; Cooper et al., 2000a). They are able to identify food using only chemical cues sampled by tongue-flicking, but little is known about the types of chemicals to which they respond or the precise chemical senses that mediate these responses. Only two recent studies document behavioral responses to certain types of food chemicals by lizards (Stanger-Hall, et al., 2001; Cooper and Pérez-Mellado, 2001a), and another study presents evidence that at least one lizard species is capable of rejecting chemically defended plant foods (Schall, 1990).

The major candidates for positive response to foods are proteins, lipids, and carbohydrates. Responses by lizards to proteins in food have not been studied, but specific glycoproteins from earthworms can elicit strong responses by garter snakes (Burghardt et al., 1988; Wang et al., 1988). The omnivorous lacertid lizard *Gallotia caesaris* responds strongly to lipids in pure pork fat and to sucrose, but the responses to these chemicals differ qualitatively (Cooper and Pérez-Mellado, 2001a). When responding to fat, the lizards tongue-flick at increased rates and frequently bite the stimulus source.

In contrast, lizards responding to concentrated sucrose solutions exhibit no elevation in tongue-flick rate or likelihood of biting (Cooper and Pérez-Mellado, 2001a). Instead, they lick the stimulus source at high rates after preliminary tongue-flicks, the rate of licking increasing with sucrose concentration. In studies of food chemical discrimination, the omnivorous lacertid *Podarcis lilfordi* licked in response to stimuli from nectar-producing flowers and licked nectar in the field (Cooper and Pérez-Mellado, 2001b), also suggesting a specific response to sugar. The only other evidence regarding responses to carbohydrates by lizards is that some individuals of the polychrotid lizard *Anolis carolinensis* preferred to eat crickets dusted with a dextrose/aspartame powder, and one licked the residue of this powder in a food bowl after eating a cricket (Stanger-Hall et al., 2001).

Evidence that lizards can use chemical cues to avoid chemically defended foods is limited to responses to the alkaloid quinine. *Cnemidophorus murinus*, an omnivore, avoids eating plants in its natural habitat that contain high concentrations of alkaloids (Schall and Ressell, 1991) and rejects experimental food that has been treated with a low concentration of quinine (Schall, 1990). Some individuals of *A. carolinensis*, an insectivore, exhibited aversive responses to crickets treated with quinine solution and showed aversive responses to crickets treated with quinine powder (Stanger-Hall et al., 2001). The signs of aversion included spitting the crickets out prior to swallowing and regurgitation. The lizards significantly avoided eating crickets treated with quinine powder.

We studied lingual and biting responses by *P. lifordi* to single representatives each of protein, lipid, and carbohydrate stimuli presented on cotton swabs. *Podarcis lilfordi* eats a wide range of invertebrate prey, occasional vertebrate food, and diverse plant species (Salvador, 1986; Pérez-Mellado and Corti, 1993). Plant parts and products consumed that are likely to contain high concentrations of sugars are fruits and nectar (Pérez-Mellado and Corti, 1993). *Podarcis lilfordi* is an excellent subject for studies of responses to types of food chemicals due to its broad diet, its abundance, and existing knowledge about its responses to food chemicals (Cooper and Pérez-Mellado, 2001b, 2001c). These lizards can discriminate between odorless and odorous control substances and surface chemicals from prey and plant foods by lingually sampling them (Cooper and Pérez-Mellado, 2001b).

We predicted that animal protein and the complex lipid mixture in pure pork fat would elicit strong responses indicated by tongue-flicking and biting and that the sucrose found in plant foods eaten by these lizards (Pérez-Mellado and Corti, 1993) would elicit licking. We also examined the effects of sucrose concentration on rates of tongue-flicking and licking and observed responses to sucrose by the lacertid species, *Lacerta perspicillata*, which is insectivorous (Barbadillo et al., 1999). The goals of the study were to assess the importance of protein to food chemical discrimination by lizards, to extend previous findings for lipids and a carbohydrate to another species, and to determine whether the licking response to sucrose was limited to species that eat plants.

METHODS AND MATERIALS

Subjects and Maintenance. Adult *P. lilfordi* were collected on the islet of Aire, offshore from Menorca, Balearic Islands, Spain, and adult *L. perspicillata* were collected in a quarry near Ciutadella on Menorca. Both were transported to a laboratory on Menorca. They were housed individually in transparent plastic terrarria ($40.5 \times 25.0 \times 26.5$ cm). The side walls of terraria were covered with white paper to reduce disturbance to the lizards by movements of the experimenters and

by other lizards. Each terrarium contained a floor of indoor–outdoor carpet and a water bowl. The natural regional light cycle was maintained by light through a window, and additional light and heat were provided by incandescent heat lamps, one suspended above the ends of each cage to permit thermoregulation. Ambient temperatures during testing were 30.9–32.2°C. Body temperatures were not measured during this experiment, but some lizards measured in these conditions at other times voluntarily attained temperatures of over 35°C, slightly warmer than the body temperature of most individuals during spring (Salvador, 1998). The lizards were fed mealworms and continuously provided water in the laboratory but were not fed for 24 hr prior to testing. All tests were conducted while lizards were active from 09:00 to 14:00 hr.

Experimental Procedures. All observations were made for responses during 60-sec trials to chemicals presented on the cotton tips of wooden applicators (15 cm). To begin a trial, an experimenter slowly moved the cotton swab to a position 1.0–1.5 cm anterior to a lizard's snout. A trial began when the lizard directed the first tongue-flick to the swab and continued for 60 sec unless the lizard bit the swab, at which time the trial was terminated. In each trial, the experimenter recorded the number of tongue-flicks, occurrence of and latency in seconds to a bite, and number of licks.

These variables and the tongue-flick attack score (TFAS) were analyzed. The tongue-flick attack score (Burghardt, 1967, 1970a; Cooper and Burghardt, 1990) is a composite variable that combines tongue-flicking and biting data to give a single index of response strength. For experiments having repeated-measures designs, TFAS(R), it is equal to the number of tongue-flicks if the lizard does not bite the swab. If a lizard bites, TFAS(R) has two components, one being based on latency to bite and the other on tongue-flicks. The term (60 minus latency to bite in seconds) is added to a term expressing a maximum number of tongue-flicks (Cooper and Burghardt, 1990). The latency term gives heavier weight to bites at shorter latency because bites at short latency indicate rapid identification of food. The other term is the maximum number of tongue-flicks by that individual in any one of its trials (Cooper and Burghardt, 1990). The tongue-flick term assures that bites are more heavily weighted than any number of tongue-flicks because bites reflect predation attempts.

In an experiment designed to detect responses to major categories of organic compounds found in prey, the stimuli tested were sucrose as a carbohydrate, pure pork fat as a complex mixture of lipids (mainly triglycerides), and bovine gamma globulin (Sigma G7516) as a protein. The fat and protein stimuli are not those from normally consumed foods. They were selected for this first study because they are readily available and inexpensive, and in preliminary trials they were found to elicit responses observed to whole foods. Sucrose is appropriate as a major contributor to the sweetness of ripe fruits and nectar although free frutose may also contribute. Deionized water was used as an odorless control stimulus.

To prepare stimuli, swabs were either dipped into deionized water or a saturated sucrose solution (6.27 M), inserted into pure pork fat, or immersed in water and then brought into contact with crystalline protein.

Twenty individuals of *P. lilfordi* were tested in a repeated-measures (randomized blocks) design. Each individual was tested in all four conditions in which the sequence of stimuli tested was varied among individuals in a partially counterbalanced order to preclude any sequential bias that might have occurred if the same sequence of stimuli were used for each individual. The minimum intertrial interval was 0.5 hr.

The effects of sucrose concentration on number of tongue-flicks, tongue-flick attack score, and number of licks were examined in an experiment having an independent-groups design in which 21 individuals of *P. lilfordi* were tested at each concentration. The concentrations of sucrose in deionized water solution were 6.27 M (saturated), 1.57 M, 0.39 M, 0.10 M, 0.02 M, 0.01 M, and 0.00 M (pure deionized water). The two highest concentrations are unlikely to be encountered in nature but are included to examine the full range of responses. Due to the independent-groups design, the tongue-flick attack score must be calculated slightly differently. TFAS is the same as TFAS(R) with the exception that the tongue-flick term for individuals that bite is the maximum number of tongue-flicks performed by any individual in the experiment. Responses by six adult *L. perspcillata* to a saturated sucrose solution were observed. We studied effects of variation in concentration only for sucrose, in part because we lacked time for additional studies during a brief visit to Menorca. In addtion, responses to protein were not as clear-cut as the licking response to sucrose, and the pork fat is a mixture of substances.

Statistical Anaylsis. Some data from both experiments were analyzed nonparametrically due to intractable heterogeneity of variance and/or nonnormality. For the experiment on responses to representatives of major categories of organic compounds, data for the continuous variables were analyzed using Friedman two-way analysis of variance. Where main effects were significant, differences among pairs of conditions were tested for significance using nonparametric multiple comparisons procedures described by Zar (1996). Data on the number of individuals that bit were analyzed using a Cochran Q test. Paired comparisons of the numbers of individuals that bit were made using sign (binomial) tests. Raw probabilities are reported for these tests, but significance tests were conducted by a sequential Bonferroni procedure to adjust for the number of tests (Wright, 1992).

Tongue-flicks in the experiment on sucrose concentration were analyzed by parametric analysis of variance for a single-factor experiment having an independent-groups design after variances were found to be homogeneous using Hartley's F_{max} tests (Winer, 1962). Paired comparisons for this variable were made using Newman-Keuls procedures. Data on number of licks from the experiment on effect of sugar concentrations were analyzed using Kruskal-Wallis analysis of

variance for continuous variables, followed by Dunn's tests for signficance of difference between pairs of sucrose concentrations when the main effect was found to be significant. An additional analysis of differences in the proportions of individulas that licked at least once between pairs of 1/4, 1/16, and 1/64 saturated solutions was conducted using Fisher exact probability tests (Zar, 1996) with Bonferroni adjustments as above. Except where indicated otherwise and justified by directional prediction, all significance tests were two-tailed, with $\alpha = 0.05$.

RESULTS

Responses to Chemical Types. All individuals of *P. lilfordi* tongue-flicked in every trial, with the greatest mean number of tongue-flicks in response to protein, but none of the stimuli elicited large numbers of tongue-flicks (Table 1). The number of tongue-flicks varied among stimuli ($\chi^2 = 8.36$, $df = 3$, $P < 0.040$). The only significant difference between pairs of conditions was that there were fewer tongue-flicks in the sucrose condition than in the deionized water condition ($P < 0.05$). There were marginally fewer tongue-flicks in the sugar condition than in the protein condition ($P = 0.063$).

The latency to bite varied among conditions, the mean being much shorter in the fat condition than in all others (Table 1). The main stimulus effect was significant ($\chi^2 = 13.61$, $df = 3$, $P < 0.004$). Latency to bite was shorter in response to fat than to deionized water ($P < 0.05$) and sucrose ($P < 0.01$). There was a nonsignificant trend for latency to bite less in response to fat than protein ($0.05 < P < 0.10$). No other differences among pairs of stimuli were significant, but latency to bite was substantially less in response to protein than to sucrose ($0.05 < P < 0.10$). Results

TABLE 1. NUMBERS OF TONGUE-FLICKS AND INDIVIDUALS THAT BIT[a]

Variable	Sucrose	Pork fat	Gamma globulin	Deionized water
Tongue-flicks				
Mean	3.1	3.9	10.2	7.0
SE	0.5	0.6	3.3	1.5
Number that bit	11	20	14	11
Latency to bite (sec)				
Mean	42.8	5.1	24.7	34.8
SE	20.7	4.5	27.0	28.7
TFAS(R)				
Mean	24.5	68.7	48.8	33.3
SE	5.1	3.3	5.0	6.4

[a] Twenty adult *Podarcis lilfordi* were used in 60-sec trials with cotton swabs bearing a saturated sucrose solution, pure pork fat, bovine gamma globulin, or deionized water.

for the number of individuals that bit swabs (Table 1) had the same pattern of significance as did latency to bite. The stimulus effect was significant ($Q = 17.25$, $df = 3$, $P < 0.001$). More individuals bit in the fat condition than in the sucrose ($P = 0.008$) and deionized water conditions ($P = 0.003$). The difference between the fat and protein conditions closely approached, but did not attain, significance after Bonferroni adjustment ($P < 0.042$ vs. adjusted $\alpha = 0.0123$). Differences among the other pairs of stimuli were not significant.

Variation among conditions in TFAS(R) was substantial (Table 1). The main effect was highly significant ($\chi^2 = 16.49$, $df = 3$, $P < 0.001$). TFAS(R) was greater in the fat condition than in the sucrose ($P < 0.05$) and deionized water ($P < 0.01$) conditions. It was also greater in the protein condition than in the sucrose ($P < 0.01$) and deionized water ($P < 0.05$, one-tailed) conditions. The remaining differences between pairs of stimuli were not significant.

Numbers of licks (Table 1) varied dramatically among conditions. Licks began only after at least one tongue-flick. Lizards licked swabs bearing sucrose rapidly, with a mean of over twice per second, but none licked swabs bearing protein. Four of 20 individuals licked swabs bearing fat. Only one individual licked a swab in the water condition, but it licked 75 times. Numbers of licks differed among conditions ($\chi^2 = 55.43$, $df = 3$, $P < 0.001$). There were more licks in the sugar condition than in the fat, protein, and deionized water conditions ($P < 0.001$ each). No other differences between pairs of stimuli were significant.

All *L. perpicillata* initially tongue-flicked swabs bearing sucrose and then licked the swabs. Numbers of licks were 36.00 ± 14.74 (SE), with range 1–103.

Sucrose Concentration and P. lilfordi. Tongue-flicks varied among sucrose concentrations ($F = 2.46$; $df = 6, 140$; $P < 0.028$; Table 2). The number of tongue-flicks in response to saturated sucrose was less than in the other conditions

TABLE 2. TONGUE-FLICKS AND TONGUE-FLICK ATTACK SCORES (TFAS)[a]

	Concentration (M)						
Variable	6.27	1.57	0.39	0.10	0.02	0.01	0.00
Tongue-flicks							
Mean	4.3	10.4	11.0	10.5	8.8	10.3	7.6
SE	0.6	2.2	2.0	1.6	1.0	1.5	0.9
TFAS							
Mean	43.9	37.2	53.7	34.2	17.4	23.6	24.2
SE	7.4	8.1	9.1	8.2	5.8	6.9	7.7
Licks							
Mean	118.4	21.0	21.3	2.4	0.0	0.0	0.0
SE	7.4	4.7	0.8	2.4	0.0	0.0	0.0

[a] Adult *Podarcis lilfordi* were used in 60-sec trials with cotton swabs bearing aqueous sucrose solutions of varying concentration. $N = 21$ for each concentration.

($P < 0.05$ each), but no other differences between pairs of concentrations were significant. TFAS differed among conditions ($F = 2.77$; $df = 6, 140$; $P < 0.015$; Table 2). The only difference in TFAS between pairs of concentrations was the greater TFAS in the 0.39 M versus 0.02 M solution ($P < 0.05$). Nevertheless, there was a trend for TFAS to be greater at the higher sucrose concentrations; the three highest TFAS scores occurred at the three highest concentrations.

The number of licks increased with the concentration of sucrose (Table 2; $\chi^2 = 100.09$, $df = 6$, $P < 0.001$). By far the greatest numbers of licks were performed in response to the 6.27 M sucrose solution, more than in all other conditions ($P < 0.05$ each). The only other difference among conditions was that the 1.57 M solution elicited more licks than each of the three solutions having the lowest concentrations ($P < 0.05$ each), none of which elicited any licking.

There were substantial differences in responses between the 1.57 M and the 0.39 and 0.10 M solutions that were not revealed in the ordinal analysis. In response to the 1.57 M solution, 14 of 21 individuals licked at least once, whereas only four and one did so in response to 0.39 M and 0.10 M solutions. A greater proportion of individuals licked in the 1.57 M group than in the 0.39 M (Fisher $P = 0.004$) and 0.10 M (Fisher $P = 0.001$) groups. A greater proportion of individuals licked in the 0.10 M group than in the combined groups at the three lower concentrations (binomial test with equal probability of licking in each group, $P = 0.008$).

DISCUSSION

Chemical Types. Podarcis lilfordi can detect and discriminate among major types of organic compounds from foods using only chemical senses. Sucrose as a representative carbohydrate, pure pork fat as a mixture of lipids, and bovine gamma globulin as a protein all elicited elevated responses by *P. lilfordi* that differed significantly among chemical types. Pork fat and sucrose elicited the strongest feeding responses; the most marked response to protein was elevated TFAS(R). It may be concluded that the lizards are capable of identifying a variety of chemicals associated with food and responding to them by investigative and feeding behaviors.

Podarcis lilfordi discriminated pork fat from sucrose and water, and exhibited a different feeding behavior in response to lipids from sucrose. Because any natural source of concentrated lipids is likely to indicate the presence of prey or plant food, feeding attempts upon detection may be adaptive even in the absence of visual cues. Lizards discriminated between bovine gamma globulin and sucrose, but there was no clear statistical evidence for discrimination between protein and pork fat. Although 70% of lizards bit in protein trials, this response was not significant because many individuals bit in all conditions. This unusual behavior for lizards may be a response to visual properties of the swabs and motion (Cooper and Pérez-Mellado, 2001b).

That protein and lipids elicit strong responses is adaptive because both are closely linked to nutritious foods. In garter snakes, specific proteins from natural prey elicit the strongest responses (Burghardt et al., 1988; Wang et al., 1988). Furthermore, some snakes tongue-flick and bite more in response to chemical cues from preferred prey than from species that are less preferred or not eaten (Burghardt, 1970b, Arnold, 1981; Cooper et al., 1990, 2000b). Response strength by lizards presumably varies among proteins within and between source species and may do so among individual lipids and lipid mixtures, emphasizing the need for future tests using chemicals from species eaten by the lizards.

After a few tongue-flicks to sucrose, lizards switched to licking, a feeding response. This change in behavior from investigation to consumption indicates rapid identification. Licking is a specific response to sucrose and likely to other sweet carbohydrates. Licking upon detecting sugar may be adaptive for omnivorous and herbivorous lizard species that frequently consume nectar or ripe fruits. The licking response was first observed for *P. lilfordi* responding to surface chemicals from nectar-producing flowers and in the omnivorous *G. caesaris* responding to a sucrose solution (Cooper and Pérez-Mellado, 2001a,b). However, that the insectivorous *L. perspicillata* licks swabs bearing sucrose hints that this behavior may be widespread in lacertids and perhaps other lizards, rather than limited primarily to omnivores and herbivores.

Licking may be an efficient means of consuming nectar and is frequently observed when *P. lilfordi* feeds on flowers in the field (Pérez-Mellado, personal observations). Occasional licks in response to pork fat suggest that licking might be a feeding response to soft or fluid substances that can be ingested without biting. In this study, excess fat could be ingested by licking. No licks occurred when excess fat was removed by wiping on a paper towel (Cooper and Pérez-Mellado, unpublished data).

Sucrose Concentration. Efforts to consume sucrose by licking appear to increase with concentration. Licking in nature would be likely to occur in response to sugars concentrated in nectar and ripe fruits. The shift from tongue-flicking to licking indicates identification of sucrose as food, which occurs more rapidly at the higher concentrations (Cooper and Pérez-Mellado, 2001a). In this, *P. lilfordi* differs from *G. caesaris*, which exhibits no variation in tongue-flick rates over a wide range of sucrose concentrations (Cooper and Pérez-Mellado, 2001a).

In a related experiment, *Gallotia caesaris* licked at a rate similar to that by *P. lilfordi* when responding to saturated sucrose but at much higher rates to 0.39 M and 0.10 M solutions (Cooper and Pérez-Mellado, 2001a). These differences might indicate that *G. caesaris* is more sensitive to sucrose or that sugar is a more important component of the diet than for *P. lilfordi*, possibly due to scarcity. Another possibility is that the two species do not differ intrinsically, and the apparent difference is an artifact of differences in experimental design. In the present study each

individual was tested only once, but each *G. caesaris* was tested at six different concentrations (Cooper and Pérez-Mellado, 2001a).

Chemical Senses. The senses that mediate food chemical discriminations by lizards are poorly known, but the major candidates are vomerolfaction, olfaction, and gustation. In garter snakes of the genus *Thamnophis* (Halpern and Frumin, 1979) and in the iguanid lizard *Dipsosaurus dorsalis* (Cooper and Alberts, 1991), vomerolfaction is required to discriminate between chemical cues from food and control chemicals. In contrast, *P. lilfordi* can locate hidden fruit using only airborne chemical cues (Cooper and Pérez-Mellado, 2001c), suggesting that olfaction also has a role in identificaiton of food by some lizards. Typically, the vomerolfactory system responds to large, nonvolatile molecules sampled by tongue-flicking (Halpern, 1992). However, vomerolfactory response to airborne volatiles cannot be excluded without experimentation.

The other chemical sense likely to participate in evaluation of food is gustation, but its function has been controversial in lizards (Schwenk, 1985). However, most lizards have taste buds on the tongue and oral mucosa (Schwenk, 1985), and substances that are sweet and bitter to humans elicit responses by *A. carolinensis* having blocked vomerolfaction (Stanger-Hall et al., 2001). Although *Anolis* lizards appear to have less well developed olfactory systems than most lizards, olfaction was not excluded in the experiment.

Some lingual behaviors provide presumptive evidence about sensory roles. When responding to sucrose, *G. caesaris* tongue-flick initially, contacting the anteroventral tip of the tongue with the swab, thereby indicating vomerolfactory sampling (Cooper and Pérez-Mellado, 2001a). They rapidly switch to licking, where the dorsal surface of the tongue that contains taste buds is brought into contact with the swab. Because licking did not occur in trials with prey surface chemicals or fat, but only in response to sucrose, it may be a specific response to carbohydrates, especially to those having a sweet taste.

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ABILITY OF HONEYBEE, *Apis mellifera*, TO DETECT AND DISCRIMINATE ODORS OF VARIETIES OF CANOLA (*Brassica rapa* AND *Brassica napus*) AND SNAPDRAGON FLOWERS (*Antirrhinum majus*)

GERALDINE A. WRIGHT, BETHANY D. SKINNER, and BRIAN H. SMITH*

Department of Entomology
Ohio State University
Columbus, Ohio 43210-1220

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Abstract—Honeybees (*Apis mellifera*) use odors to identify and discriminate among flowers during foraging. This series of experiments examined the ability of bees to detect and discriminate among the floral odors of different varieties of two species of canola (*Brassica rapa* and *Brassica napus*) and also among three varieties of snapdragons (*Antirhinnum majus*). Individual worker honeybees were trained using a proboscis extension assay. The ability of bees to distinguish a floral odor from an air stimulus during training increased as the number of flowers used during training increased. Bees conditioned to the odor of one variety of flower were asked to discriminate it from the odors of other flowers in two different training assays. Bees were unable to discriminate among flowers at the level of variety in a randomized presentation of a reinforced floral odor and an unreinforced floral odor. In the second type of assay, bees were trained with one floral variety for 40 trials without reinforcement and then tested with the same variety or with other varieties and species. If a bee had been trained with a variety of canola, it was unable to differentiate the odor of one canola flower from the odor of other canola flowers, but it could differentiate canola from the odor of a snapdragon flower. Bees trained with the odor of snapdragon flowers readily differentiated the odor of one variety of a snapdragon from the odor of other varieties of snapdragons and also canola flowers. Our study suggests that both intensity and odor quality affect the ability of honeybees to differentiate among floral perfumes.

Key Words—*Apis mellifera*, *Brassica napus*, *Brassica rapa*, *Antirhinnum majus*, proboscis extension assay, floral odors, olfactory learning, honeybee.

* To whom correspondence should be addressed. E-mail: smith.210@osu.edu

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INTRODUCTION

The scent of a flower is a mixture of many volatile compounds that varies with respect to genotype, stage of development, and local environmental conditions (Dobson, 1994; Dudareva and Pichersky, 2000). The number, structure, and concentration of volatile chemicals emitted by individual flowers vary substantially among species of plants (Dobson, 1994; Dudareva and Pichersky, 2000). Genetic variation within a species may also modify the amount and type of compounds in the odor profile, producing varieties that are perceptually different to humans. This has been shown for the rose, *Rosa hybrida* (Dobson et al., 1987; Kim et al., 2000), oilseed rape or canola (Robertson et al., 1993), and other species of plants including snapdragons (*Antirhinnum majus*) (Dudareva et al., 2000), *Narcissus* (Dobson et al., 1997) and *Clarkia brewerii* (Nam et al., 1999). Flowers common to the same plant may also exhibit quantitative differences in volatile compounds (Schiestl et al., 1997; Dudareva and Pichersky, 2000) that vary diurnally and developmentally (Faegri and van der Pijl, 1979; Matile and Altenburger, 1988; Tollsten and Bergstrom, 1988; Jakobsen and Olsen, 1994; Dudareva et al., 2000) and with respect to their pollination status (Tollsten and Bergstrom, 1989; Schiestl et al., 1997).

This amount of variation in floral odorants is likely to have evolved to attract specific pollinators to flowers (Faegri and van der Pijl, 1979; Pellmyr and Thien, 1986; Metcalf, 1987; Dobson, 1994; Olesen and Knudsen, 1994). Pollinators, such as honeybees, use odors to discriminate among flowers while foraging (Waller et al., 1974; Pham-Delegue et al., 1986). Honeybees forage by selecting flowers that have similiar shape, color, and odor (Free, 1963; Chittka et al., 1999). Flowers are often accepted or rejected based ultimately on their odor (Faegri and van der Pilj, 1979; Dobson, 1994). Odors improve the floral constancy of honeybees by providing them with specific additional cues that aid in flower identification and memory (Dobson, 1994; Chittka et al., 1999). However, each individual flower may not be identical to other flowers of the same species or even others on the same plant. After feeding from a nutritionally acceptable flower, bees must be able to generalize among the odor cues presented by flowers of the same species. They must also be able to differentiate their target flower from others present in the environment. Their ability to distinguish acceptable flowers from nutritionally unacceptable flowers will directly affect the efficiency and fitness of the colony of honeybees (Chittka et al., 1999) and may also affect the fitness of the plants they pollinate (Odell et al., 1999).

It is important, therefore, to examine the characteristics of a flower's odor that individual honeybees use to identify acceptable flowers. Can bees discriminate among flowers that contain the same compounds in different concentrations, or is it essential that the odor profile contain compounds that are different from one another for bees to efficiently discriminate among flowers? To begin to answer these questions, we chose to examine the olfactory learning behavior of honeybees when

they were trained to respond to two types of well-studied flowers: canola flowers (*Brassica rapa* and *Brassica napus*) and snapdragons (*Antirhinnum majus*). As much is known about honeybee olfactory learning, we used the honeybee as a model generalist pollinator, even though it does not often forage on snapdragons, as the individual flowers are too difficult for workers to open. Both types of flowers have distinct odors, and the volatile components of the perfumes of both the flowers of canola (Tollsten and Bergstrom, 1988; Robertson et al., 1993; Butcher et al., 1994; Jakobsen et al., 1994; Blight et al., 1997; McEwan and Smith, 1998; Omura et al., 1999) and the flowers of snapdragons (Odell et al., 1999; Dudareva et al., 2000) have been previously identified and quantified. In addition, varieties of both types of flowers have been shown to contain many of the same compounds present at different concentrations (Roberston et al., 1993; Odell et al., 1999). A given variety may also contain compounds that are unique to that variety. Both types of variation in odor composition produce unique perfumes, so these differences could have large impacts on the ability of bees to differentiate among the odors of each type of flower.

Presently, an extensive literature has developed about the learning and discrimination of pure odors and simple mixtures by honeybees (Menzel, 1990; Smith, 1998; Chandra et al., 2000; Chandra and Smith, 1998). Our objective in this study is to apply the same types of conditioning procedures used previously to evaluate how honeybees discriminate natural floral perfumes (Pham-Delegue et al., 1989, 1993, 1997). This type of research ensures that the results generated from laboratory studies of pure odorants generalize to studies of honeybees and their olfactory learning in their natural environment. In the present study, we trained individual bees to the scent of one variety of our chosen floral species and then examined their responses to the same variety and to different varieties. With this type of assay, we tested the ability of bees to discriminate and/or generalize among the odors of different flowers after conditioning with one flower type. We were particularly interested in examining generalization with respect to inter- and intraspecific relatedness of the flowers and the similarity of their floral perfumes.

METHODS AND MATERIALS

Flowers. Two varieties (from Svalöf Weibull Seed Ltd., Saskatoon, Saskatchewan, Canada) of each canola species, *Brassica rapa* and *Brassica napus*, were grown over a two-month period between May and July 1999 or June and August 2000 in a field at the Waterman Agricultural and Natural Resources Station, Ohio State University. Two varieties of *B. rapa*, Horizon and Valleyview (Svalöf Weibull Ltd.), hereafter referred to as BR1 and BR2, and one variety of *B. napus*, Bullet (Svalof Weibull Ltd.), hereafter referred to as BN, were selected for testing. The canola flowers were isolated from pollinators in the field by 1.3- $\times$ 1.3-m

exclosures covered with screening to keep worker bees from pollinating the flowers. The exclosures were moved periodically to randomize sampling across the flowers. Inflorescences were collected in the morning and early afternoon, and the stems were placed in water. Selection of flowers from the inflorescences was random, but all flowers selected were fully opened.

Three varieties of ornamental snapdragon (*Antirhinnum majus*) studied at Purdue University (Dudareva et al., 2000; Murfitt et al., 2000; Kolosova et al., 2001) were grown in the greenhouse from April 2000 from plants obtained from N. Dudareva. These varieties were called Pale hybrid, Potomac Pink, and Maryland True Pink (henceforth PH, PP, and MTP). Previous studies of volatile emissions from these hybrids and other snapdragons have indicated that volatile emissions peak for this species of flowers on the fifth day after anthesis (Dudareva et al., 2000). Therefore, flowers chosen for training bees were between four and six days after anthesis. The flowers were collected in the morning on the day of testing.

The canola flowers were clipped by the petiole from inflorescences and placed in new 10-cc glass syringes. The syringes were modified to deliver the headspace volatiles from the flowers by attaching the needle end via 1/16-in. tubing to a mini-solenoid valve (Lee Co.) that controlled air flow from an aquarium air pump (Tetratec DW 96-2). The syringe was closed on the open end with a silicon stopper that had a modified, glass Pasteur pipet forced through the middle. The snapdragon flowers were placed in an Erlenmeyer flask with a tubulation arm, and the opening of the flask was fitted with a modified silicon stopper. The silicon stopper had a glass Pasteur pipet forced through it that was attached to an air supply via tubing in the same manner as the syringes for the canola flowers. The tubulation arm was modified by excising the narrow tip of a Pasteur pipet from the rest of the pipet and attaching it to the tubulation arm with putty. The narrow end of the pipet from both the flasks and the syringes faced outward, and it was aimed approximately 3–4 cm from the bee testing platform. Bees were unable to view the flowers within the syringe and the flasks. Both types of odor delivery device were washed three times with distilled, deionized water after use and allowed to dry, and each was specifically reused for the same variety of flower each time.

Honeybees. Individual bees were collected over two summers: 1999 and 2000. During the 1999 season, worker honeybees (*Apis mellifera*) were collected from the hive entrance as they left to forage between 8 and 10 AM. They were trapped individually in glass vials and cooled on ice. When they were quiescent, each bee was placed individually in a restraining harness as described by Bitterman et al. (1983) and by Smith (iris.biosci.ohio-state.edu/honeybee). Each bee was fed 0.4 μl of a 1.25 M sucrose solution and left for 2 hr. During the 2000 season, worker honeybees were collected from the hive entrance as they were leaving to forage between 1 and 5 PM. Each bee was fed 2–3 μl of a 1.5 M sucrose solution and left on the laboratory bench at 22°C overnight. The next morning, the bees were tested for motivation by touching one antenna with sucrose and observing

the subsequent proboscis extension. Bees that did not extend their proboscis were not used in the experiment.

Conditioning Protocol. Honeybees readily learn to associate odorants with sucrose reinforcements (Bitterman et al., 1983; Menzel, 1990). All experiments utilized the proboscis extension response (PER) assay to evaluate the olfactory learning behavior of individual bees restrained as described above. Three different experimental protocols were used to test their ability to detect and discriminate floral volatiles.

Relative Detectability of Odor of Different Floral Varieties. Bees were placed in a conditioning arena and conditioned to discriminate a floral odor from an air background stimulus. Individual bees were trained as described in Smith (1998), with an intertrial interval of 5 min. Each bee received conditioning to the odor of one species of flower in the following pseudorandomized, 12 trial sequence: F – A – A – F – A – F – F – A – F – A – A – F, where F = flower and A = air (Smith et al., 1991). The floral odors were paired with a 0.4-μl droplet of 1.5 M sucrose. The air stimuli were not reinforced. On a given trial, each bee was recorded as having responded if it extended its proboscis during the first 3 sec of odorant delivery.

Each of the varieties of canola was separated into three treatments: 1 flower per syringe, 5 flowers per syringe, and 10 flowers per syringe. This arrangement was designed to test if differences in concentration of odor were detected by the bees and reflected in their ability to discriminate the odor stimulus from the air stimulus. Snapdragon flowers were tested with only one flower per flask.

Discrimination of Floral Odors. Two protocols were employed to examine the ability of bees to discriminate among floral odors from the species and varieties of canola and snapdragon chosen for this experiment. The first utilized the same 12 trial, pseudorandomized sequence as above. One floral odor was paired with sucrose and the other was not reinforced. The following pairs of flowers were tested, with the underlined species of each pair receiving reinforcement: Pair 1: BR2 × BR1; Pair 2: BR1 × BN; Pair 3: BN × BR2; Pair 4: PH × BR1; and Pair 5: PH × PP. The responses of individual bees to each exposure of odor was scored as above.

The second means of examining discrimination utilized a paradigm developed to study latent inhibition in honeybees (Chandra et al., 2000). This protocol exposes individual bees to 40 unreinforced training trials with an odorant, during which a bee is stimulated with an odor, but no sucrose reinforcement is given. We switched to this conditioning paradigm because we could make use of an automated, odorant delivery system described in detail in Chandra et al. (2000). The automation facilitated delivery of a large number of trials. After the 40 training trials, each bee was tested in a seven-trial acquisition test with either the same odorant or a different odorant. Each trial presented the odorant reinforced with 0.4 μl of 1.5 M sucrose.

In this training paradigm, repeated presentation of a specific odorant over 40 trials without reinforcement leads to a slower than normal rate of learning to that odorant during the posttraining test phase (Chandra et al., 2000). In essence, the bees learn to ignore the stimulus during the pre-training phase. Alternatively, if a bee is pre-trained with one odorant and then post-tested with a novel odorant that is perceptually different from the original odorant, the rate of learning to the novel odorant should not be affected by the previous training with the original odorant (Chandra et al., 2000). We used this technique with the floral odors with the expectation that bees would show faster rates of acquisition during the test phase to a different test flower if they could readily differentiate the odor of a test flower from the odor of the training flower.

In this experiment, the delivery of the floral odor was the same as for the forward pairing, utilizing a syringe for canola flowers and an Erlenmeyer flask for snapdragons. In the canola treatments, the syringes contained 10 flowers each and were replaced with fresh flowers every 50 min. In the snapdragon experiment, the flasks contained three flowers, and they were also replaced every 50 min. During the acquisition trials, a separate syringe or flask was used to test each individual bee. The bees were "inhibited" to one species of a flower's odor, and then tested with one of four treatments: the same flower species; the same species, but a different variety; a different species; and a species from a different family. These treatments were used for all species of canola; for the snapdragons, only the PH variety was inhibited, and it was tested against itself, two other varieties, and *B. rapa* 1. In this way, we sought to examine the bees' ability to discriminate floral odors across different taxonomic levels.

Statistical Analyses. For the forward pairing experiment, the total number of responses for each stimulus was summed, and the sum for the unreinforced air stimulus was subtracted from the sum for the reinforced floral odor. This difference was tested in a Student's t test against the expected value of zero given an equal number of responses to odor and air (i.e., the odor was not detectable). This was repeated across the species for each of the treatments of 1, 5, and 10 canola flowers versus the air stimulus and the snapdragon versus the air stimulus. As this test was calculated for 10 different treatments at the $\alpha = 0.05$ level, a Bonferroni method of controlling for experiment-wise error rate was used to evaluate the significance levels of each of the comparisons (Sokal and Rohlf, 1995), where $\alpha'' = \alpha/n$, and n is the number of comparisons.

This type of analysis was also used to evaluate the same training procedure used for the simple discrimination test, where one floral odor was paired with sucrose and the other was unreinforced.

For the experiment in which each species of canola was inhibited in the latent inhibition protocol, the responses of each individual bee for each trial was summed at the end of the seven acquisition trials for each bee. A separate one-way ANOVA was employed to examine the differences in the sums among the treatments after

TABLE 1. RESULTS OF *t* TESTS FOR DIFFERENCE BETWEEN HONEYBEE RESPONSE TO FLORAL ODOR VERSUS AIR, $H_{null} = 0$

	B. rapa 1			*B. rapa* 2			*B. napus*		
Flowers/treatment	*df*	*t*	*P*	*df*	*t*	*P*	*df*	*t*	*P*
1	9	2.22	0.053	11	2.60	0.025	11	1.34	0.206
5	9	4.69	0.001[a]	11	3.12	0.010	11	4.24	0.001[a]
10	9	4.94	0.001[a]	11	3.87	0.003[a]	11	7.15	<0.001[a]

[a] Significant after comparison to a Bonferroni adjusted significance level ($P < 0.005$).

inhibition for each flower used during training (Sokal and Rohlf, 1995). The least-squares differences were compared for the responses to the test flower versus the responses to the training flower (Sokal and Rohlf, 1995).

RESULTS

Relative Detectability of Odor of Different Floral Varieties. Bees were unable to discriminate any of the canola odors from air when only one flower was present in a syringe (Table 1, Figure 1). They were able to discriminate the odor of BR1 and BN from air when 5 and 10 flowers were placed in each syringe (Table 1, Figure 1). They were only able to discriminate BR2 from air for the 10 flower treatment (Table 1). In contrast, the odor of a single snapdragon flower appeared to be easily discriminated from the air stimulus for each snapdragon variety tested (Table 2, Figure 2).

Discrimination of Floral Odors. In the discrimination protocol, the bees were unable to discriminate the odor of BR2 from BR1 ($df = 11, t = -1.86, P = 0.09$) (Figure 3a), BR1 from BN ($df = 11$, $t = 0.00$, $P = 1.0$) (Figure 3b), BN from BR2 ($df = 9$, $t = -0.81$, $P = 0.434$) (Figure 3c), or PH from PP ($df = 9$, $t = 0.88$, $P = 0.399$) (Figure 3d). The only pairing of flowers that bees were able to discriminate in this paradigm was PH with BR1 ($df = 9$, $t = 3.28$, $P < 0.009$) (Figure 3e).

TABLE 2. RESULTS OF *t* TESTS FOR DIFFERENCE BETWEEN HONEYBEE RESPONSE TO FLORAL ODOR VERSUS AIR[a]

Pale hybrid			Potomac Pink			Marilyn True Pink		
df	*t*	*P*	*df*	*t*	*P*	*df*	*t*	*P*
14	9.40	<0.001	11	6.38	<0.001	11	8.68	<0.001

[a] $H_{null} = 0$, one flower per treatment.

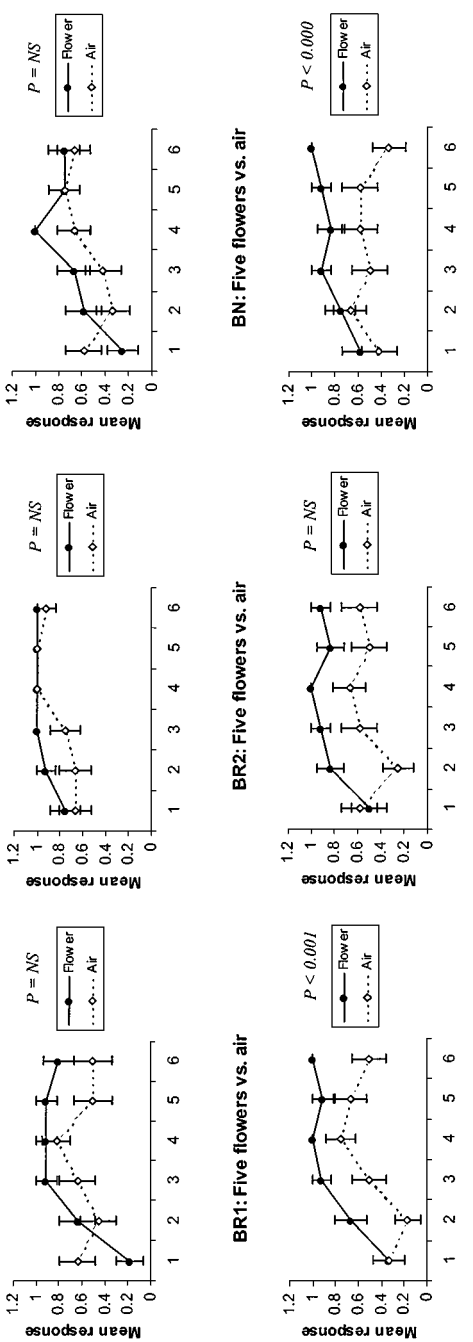


FIG. 1. PER (mean ± SE) of honeybees ($N = 12$ per graph) on each trial during 12 trials for different numbers of canola flowers per syringe. Flower treatments were rewarded with 0.4 μl of 1.5 M sucrose every trial; air treatments were unrewarded. P values are derived from a Student's t test where $H_{\text{null}} = 0$.

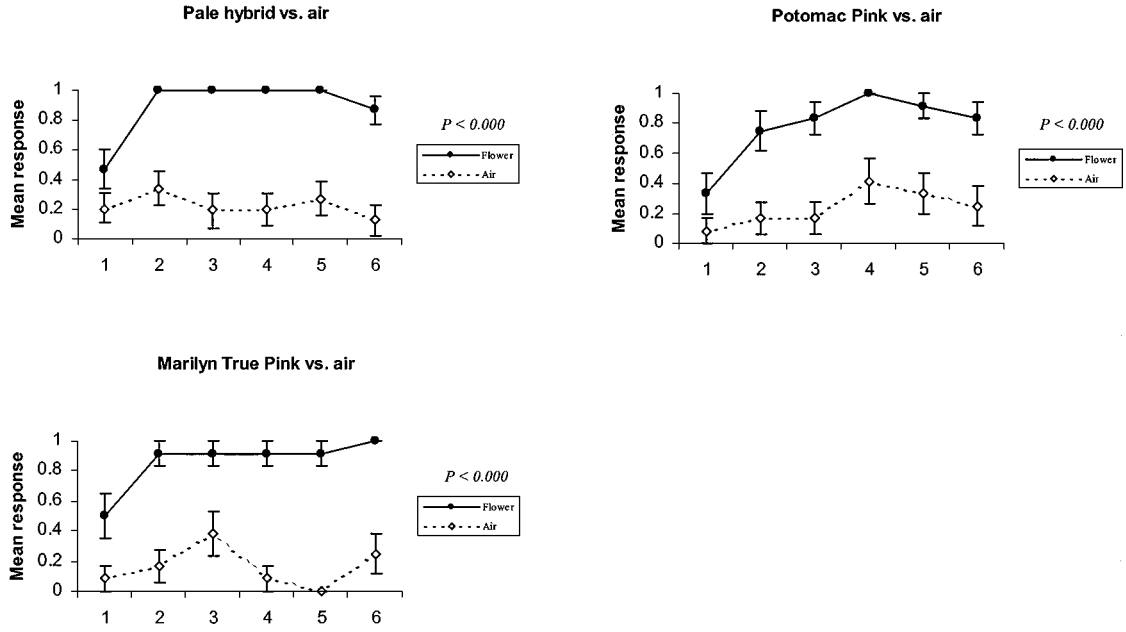


FIG. 2. PER (mean ± SE) of honeybees ($N = 30$) on each trial during 12 trials for pale hybrid snapdragons (one flower per flask). Flower treatments were rewarded with 0.4 μl of 1.5 M sucrose every trial; air treatments were unrewarded. P values are derived from a Student's t test where $H_{\text{null}} = 0$.

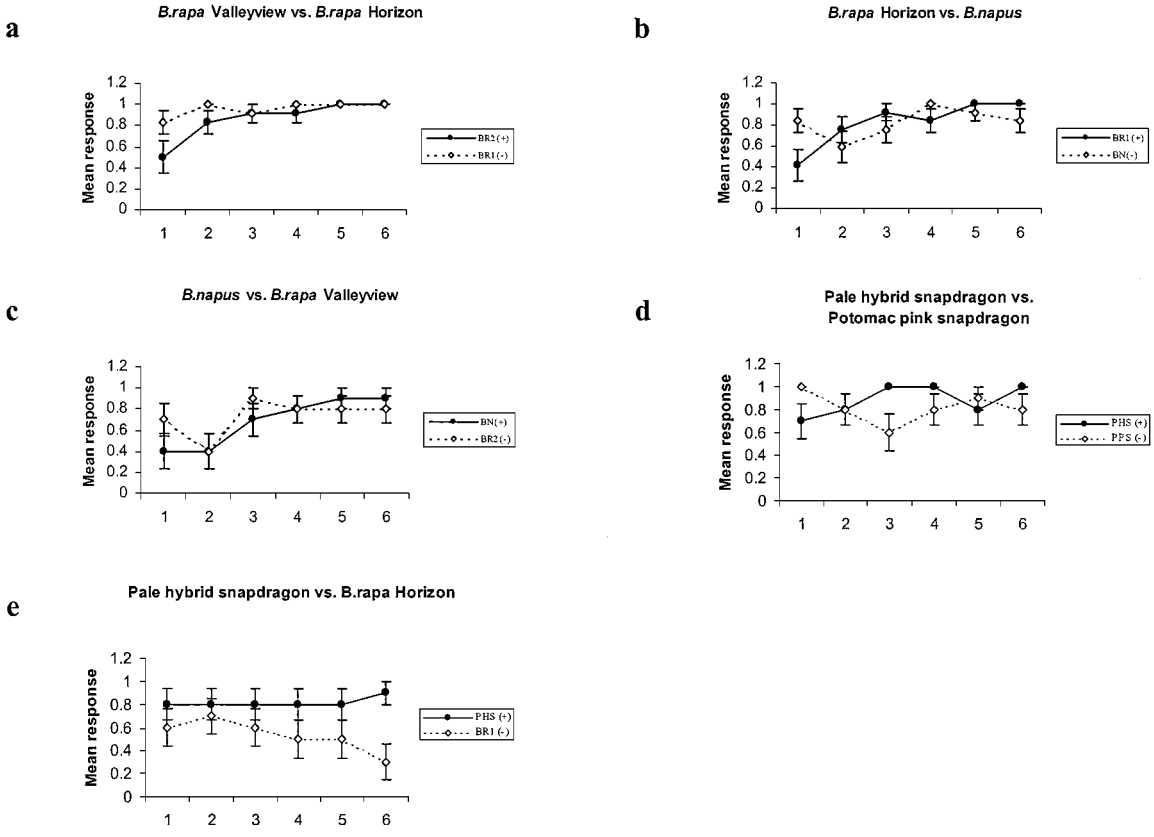


FIG. 3. PER (mean ± SE) of honeybees ($N = 10$/group) on each trial during 12 trials. Flower treatments in black were rewarded with 0.4 μl of 1.5 M sucrose every trial (+); flower treatments in white were unrewarded (−).

TABLE 3. RESULTS OF ONE-WAY ANOVA FOR INHIBITED FLOWER VERSUS TEST FLOWER FOR *B. rapa* 1, *B. rapa* 2, *B. napus* AND PALE HYBRID SNAPDRAGON

	Inhibited flower type											
	B. rapa 1			*B. rapa* 2			*B. napus*			PH snapdragon		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Between groups	3, 111	3.73	0.013	3, 142	1.06	0.367	3, 165	2.78	0.044	3.80	5.87	0.001
	Least-squares differences											
	Test flower		*P*	*Test flower*		*P*	*Test flower*		*P*	*Test flower*		*P*
	BR2		0.309	BR1		0.769	BR1		0.094	PP		0.001
	BN		0.581	BN		0.568	BR2		0.106	MTP		0.009
	Snapdragon		0.002	Snapdragon		0.241	Snapdragon		0.005	BR1		0.006

The latent inhibition protocol showed a similar trend for the canola species. Separate one-way ANOVAs indicated that responses to the test flowers during the seven post-training acquisition trials were different when either BR1 or BN was used to produce inhibition (Table 3, Figure 4). The inhibited flower had the smallest response during the test trials in all cases (Figure 4), which is consistent with previous studies using this technique (Chandra et al., 2000). The other varieties and species of canola had slightly greater responses during the test, and the snapdragon odor provoked the greatest number of responses (Figure 4). The comparison of the inhibited species to each of the other tested species (Table 3) failed to reveal a difference among the inhibited canola species/varieties and the tested canola species/varieties (Table 3), indicating that individual honeybees could not easily discriminate among these varieties. The responses during the test after inhibition to BR1 and BN were significantly different only for the comparison between the inhibited odor and the snapdragon odor.

A one-way ANOVA for the PH snapdragon also demonstrated that the responses across each species during the test were significantly different from the inhibited flower ($df = 3, 80$, $F = 5.87$, $P < 0.001$) (Figure 4). This occurred in the same pattern as was seen for the canola, where the inhibited flower showed the lowest response, the other varieties of snapdragon a slightly greater response, and the canola flower provoked the greatest response during the test (Figure 4). The least-squares difference confirmed that significant differences occurred for the comparisons of the inhibited odor to each of the test flower odors (Table 3). Therefore, it appeared that individual honeybees could discriminate among the odors of each variety of snapdragons and the canola variety, BR1, after conditioning to the pale hybrid snapdragon.

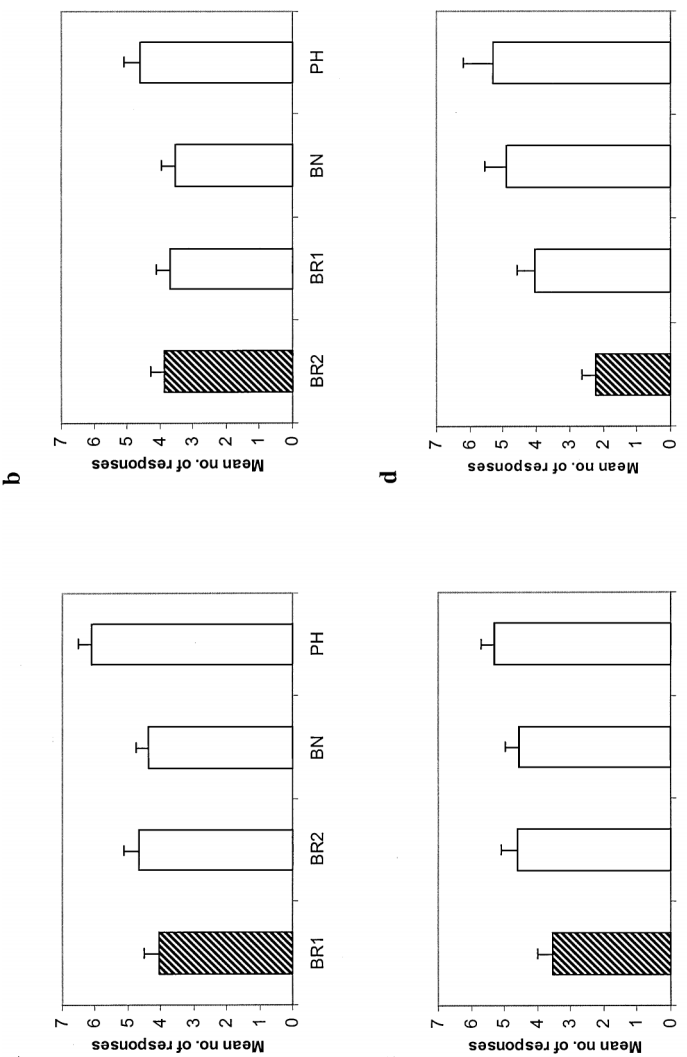


FIG. 4. PER (mean + SE) of honeybees to four different flower types during seven test trials after inhibiting to one flower type (the striped bar indicates the inhibition flower). Ten flowers per treatment were used for each canola variety; three flowers per flask were used for snapdragons: (a) inhibition to *B. rapa* 1 ($N = 112$), (b) inhibition to *B. rapa* 2 ($N = 160$), (c) inhibition to *B. napus* ($N = 143$), and (d) inhibition to pale hybrid snapdragons ($N = 84$).

DISCUSSION

Detectability and Discriminability of Floral Odors. The intensity of an odor stimulus produced by flowers may play an important role in initially attracting pollinators and in causing them to seek out perceptually similar flowers (Faegri and van der Pilj, 1979; Dobson, 1994). Many plant species produce floral arrays where individual flowers are arranged closely together, perhaps to influence pollinator perception and recognition among species of plants by increasing a given plant's impact on an animal's sensory apparatus. In addition, some species of plants have individual flowers that produce odor volatiles in greater concentrations than the individual flowers of other plant species (Dobson, 1994). Evidence from laboratory-based experiments suggests that odorant concentration affects both the detection and memory of odorants by honeybees, as more intense odors are generally learned faster and are more readily distinguished from other odors (Bhagavan and Smith, 1996; Pelz et al., 1997). Until now, the ability of bees to learn the odors of living flowers at natural intensities has not been often studied (Gerber et al., 1996).

To examine the effect of flower odor intensity on the rate of acquisition by honeybees, we trained individual bees to the odor of 1, 5, and 10 canola flowers. Our first experiments examined the ability of honeybees to discriminate the scent of *B. napus* or *B. rapa* flowers paired with sucrose reinforcement from an unreinforced, "background" air stimulus. We designed the first set of experiments as an assay of detectability, where we expected that if the stimuli represented by the flowers of the same unpollinated, developmental stage were approximately equal, then the odorant stimulus would increase in proportion to the number of flowers used.

The acquisition curves for honeybees showed that discrimination of the floral odor stimulus from the air stimulus increased as the number of flowers per treatment increased. When 10 flowers were placed in each modified syringe, the bees could clearly distinguish between a floral odor stimulus and an air stimulus for each variety. This suggests that 10 flowers of each variety produce a stimulus that is above the threshold of detectability for honeybees. At the level of one flower per syringe for each variety of canola, no difference is seen between the acquisition curves for floral odor versus air. Differences in detectability among varieties began to occur at the level of five flowers per syringe at which the second variety of *B. rapa* was not significantly distinguished from an air stimulus. This may be due to a weaker concentration of odor produced by these flowers, as the other two varieties were detectable when five flowers were present. We repeated this experiment with one snapdragon flower per flask for each variety of snapdragon; it was clear for all the varieties that the odor produced by a single flower was strong enough for them to discriminate the floral odorant from an air stimulus. A comparison of the responses to canola flowers with the responses to snapdragon flowers indicates that snapdragons produce odors that are more detectible than

individual canola flowers, as the threshold for detectability for a given snapdragon flower was reached using only one flower, regardless of variety.

We then examined the ability of honeybees to differentiate flowers of one variety reinforced with sucrose from flowers of different varieties that were not reinforced. Bees were unable to discriminate between flowers of different varieties and among the different species of canola (Figure 3a–d). However, when asked to differentiate the odor of a snapdragon flower from the odor of canola flowers, they successfully learned the discrimination task. Therefore, in general, the discrimination of floral varieties of *Brassica* was not detectable in this assay. The only case in which bees were able to discriminate among the odors of different floral varieties was when they were asked to choose between the odor of a snapdragon flower and the odor of a canola flower.

Previous experiments using pure odorants in this same 12-trial assay demonstrated that honeybees can easily discriminate different odors (Menzel, 1990). Lack of discrimination in our use of this assay may only imply that the discrimination task required of the bees is difficult. In the field, individual worker honeybees may experience hundreds of flowers on a single foraging trip. With more experience (i.e., more exposures to a specific target conditioned stimulus), honeybees might demonstrate the ability to discriminate among the odors of similar flowers.

To address this possibility, we decided to utilize an assay that repeatedly presented the odor of a particular flower 40 times without reinforcement (Chandra et al., 2000). In this assay, individual bees are exposed to a discrete odor stimulus 40 times at 5-min intervals. The bees are then tested during a post-test acquisition phase, during which the same or a different odor stimulus is presented with reinforcement. The mean response to each type of test stimulus can then be compared.

We expected that the rate of acquisition during the test phase would be proportional to the similarity of the odor of the test flower to that of the training flower. If a novel odor is perceptually similar to the pre-exposed odor, then it will be learned more slowly than a perceptually dissimilar odor. When bees were pre-exposed to the odors of BR1, BR2, or BN, the acquisition responses were lowest during the test phase for each of these flower types, which is consistent with Chandra et al. (2000). The lack of significantly faster acquisition to the other canola species and varieties indicates that they were not easily discriminable from any given pre-exposed variety of canola in this assay. Thus, in general, the *Brassica* species used in our experiments are relatively difficult to discriminate, even though the odors they produced are all detectable by honeybees.

Not surprisingly, *Brassica* odors are discriminable from snapdragons; the response to the odor of a snapdragon was significant for BR1 and BN. Interestingly, none of the responses during the test were significantly different for the individuals inhibited with BR2. As shown in the previous section, intensity of an odor stimulus, at least as indexed by the number of flowers, can strongly affect the ability of bees to discriminate among odors. The rate of acquisition of individual bees to five

flowers of BR2 during the training was slower than the rate of acquisition of five flowers of either BR1 or BN. This suggests that the odors of BR2 were not as intense as the odors of the other flowers used during training. The inability of bees to discriminate BR2 and the snapdragon flowers during the test after pre-exposure with BR2 also supports the idea that the odor of BR2 flowers was less intense than the odor of the other flowers.

When bees were inhibited with the odor of the PH snapdragon, they clearly demonstrated the ability to discriminate among other snapdragons and other species of flowers during the test. Thus, individual flowers of this group are relatively more discriminable than was the case for the *Brassica* species. This could be a result of both the intensity of the odor produced by the snapdragons and the types of compounds found in the floral perfume. The intensity of the snapdragons appeared to be greater than the intensity of the canola flowers, as indicated by our assay of the rate of learning of individual honeybees. Differences in the intensity and/or quality of the floral perfumes may therefore have allowed bees to differentiate among the snapdragons more readily during the test phase after pre-exposure to the odor of the pale hybrid snapdragon.

Qualitative Variation in Floral Volatiles. The discriminability of floral varieties is likely to be related to the chemical composition of their odors. The volatile compounds of both of the floral types chosen for this series of experiments have been identified in previous studies. The main types of compounds reported from the *Brassica* species are organic sulfides, C_4 substituted compounds, aliphatic and aromatic aldehydes, and mono- and sesquiterpene hydrocarbons (Robertson et al., 1993). Short-chain alcohols, ketones and acids were also detected (Robertson et al., 1993).

One report of the volatiles of five varieties of *B. napus* showed that they contain about 75–83% of compounds in common (Robertson et al., 1993). Comparisons across studies have revealed that qualitative differences are greater between *B. napus* (Tollesten and Bergstrom, 1988; Robertson et al., 1993; Butcher et al., 1994; Jakobsen et al., 1994; Blight et al., 1997; McEwan and Smith, 1998) and *B. rapa* (Omura et al., 1999). Six of 97 compounds from *B. rapa* (sum reported from all studies of *B. napus* cited before) were reported in common with *B. napus* (Omura et al., 1999). However, the method used to sample floral headspace significantly affects the amount and type of compounds reported (Dobson, 1994; Agelopoulous and Pickett, 1998; Raguso and Pellmyr, 1998), and the sampling procedures were quite different among all the studies examined. More compounds might be found in common among the species if a comparison were made of their volatiles using the same sampling procedures.

Snapdragons have been reported to contain substantially fewer compounds than canola flowers. The following constituents have been reported and identified from all of the varieties that we studied: methyl benzoate, *trans*- and *cis*-β-ocimene, myrcene, linalool, *trans*- and *cis*-methyl cinnamate, *cis*-3-hexenyl

acetate, acetophenone, dimethoxytoluene, diphenyl ether, and nerolidol (Odell et al., 1999; Dudareva et al., 2000; Murfitt et al., 2000; Kolosova et al., 2001). Other minor components have not yet been identified (Dudareva, personal communication).

Qualitative differences occur in both the species and varieties of canola flowers and in the varieties of snapdragons. The only compounds reported in common among snapdragons and canola flowers are *trans*-β-ocimene, myrcene, and linalool from *B. napus* (Robertson et al., 1993) and from some varieties of snapdragons (Odell et al., 1999; Dudareva et al., 2000). It is clear from our discrimination assay that bees have difficulty distinguishing among canola flowers; it is also clear that they were able to distinguish among snapdragon varieties. The differences in detectability and discriminability seen for these two types of flowers must be related to volatiles given off by the flowers. Previous hypotheses about the selection behavior of foraging honeybees propose that bees select flowers based on their ability to detect and respond to individual volatile components of a floral perfume (Pham-Delegue et al., 1986, 1989, 1993, 1997; Henning et al., 1992; Wadhams et al., 1994; Blight et al., 1997; Le Métayer et al., 1997). These studies typically isolate the volatiles from flowers, separate them into fractions, and then assay the response of both free-flying or restrained honeybees. This type of analysis predicts that certain compounds found in flowers, including canola and sunflowers, are more attractive and more easily learned by honeybees than others present in the perfume. It would also predict that differentiation among floral perfumes would result from qualitative differences among perfumes.

We were particularly interested in examining the ability of bees to discriminate among flowers that may exhibit subtle differences in their entire perfume. Our results for the tests of the ability of bees to discriminate the varieties of snapdragons show that flowers that have the same volatile components can be perceptually different. Other studies of bee discrimination among perceptually similar flowers have suggested that bees can distinguish among flowers that produce all of the same compounds, where each compound is present at slightly different concentrations in the floral headspace of individual flowers or plants (Beker et al., 1989; Ayasse et al., 2000). An experiment by Beker et al. (1989) tested the ability of free-flying bees to discriminate among the varieties of *Majorana syriaca*, and also showed that they were able to distinguish among different varieties, even though the chemical components of the perfumes of both varieties were mostly the same (Dudai et al., 1988; Beker et al., 1989). The solitary bee, *Andrena nigroaenea*, was shown to differentiate among individual flowers of the orchid, *Ophrys sphegodes*, based on variation in the quantities of the odor components found among individual flowers (Ayasse et al., 2000). In this case, the variation of individual odor components caused subtle differences in the floral perfume from flower to flower (Schiestl et al., 1997; Ayasse et al., 2000), which the bees apparently perceived as unique to individual flowers, and so were deceived into pollinating the orchid without

reinforcement. The inability of bees to distinguish *Brassica* floral odors from each other suggests that although they may have only 75% of their compounds in common, their odors are perceptually similar to honeybees. Thus, the perceptual similarity of the perfumes of flowers may not only be related to the number of compounds in common, but may also be a function of the relative concentration of certain key components of the floral odor (Pham-Delegue et al., 1989, 1993; Blight et al., 1997).

Differentiation by Honeybees among Floral Perfumes. Our study suggests that both intensity and odor quality affect the ability of honeybees to differentiate among floral perfumes. Other factors may also influence discrimination. The general intensity of the floral perfume and the intensity of individual odor components may contribute equally to the differences in perceptual qualities that arise among similar flowers. In addition to the contribution of the presence of individual compounds in a perfume, the number of compounds and the chemical similarity of the compounds may also affect the ability of bees to discriminate among flowers.

Studies of honeybee olfactory learning behavior typically do not utilize live flowers; indeed, few studies have examined honeybee learning and discrimination at concentrations that are similar to the intensities encountered in individual flowers (Pelz et al., 1997). Training honeybees to discriminate among odors presented at biologically relevant concentrations rigorously examines their true ability to differentiate among floral odors. Further examination of the characteristics of the odors of individual flowers may provide clues about which features of the odors of individual flowers bees learn and remember.

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INFLUENCE OF ODOR FROM WOOD-DECAYING FUNGI ON HOST SELECTION BEHAVIOR OF DEATHWATCH BEETLE, *Xestobium rufovillosum*

STEVEN R. BELMAIN,[1,†] MONIQUE S. J. SIMMONDS,[2,*] and WALLY M. BLANEY[1]

[1]*School of Biological & Chemical Sciences*
Birkbeck College, University of London
Malet Street, London WC1E 7HX, United Kingdom

[2]*Jodrell Laboratory*
Royal Botanic Gardens
Kew, Richmond, Surrey TW9 3AB, United Kingdom

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Abstract—Adult females of *Xestobium rufovillosum* de Geer demonstrated anemotactic orientation when exposed to an odor plume containing volatiles generated by wood-decaying fungi (*Coriolus versicolor*, *Donkioporia expansa*) and decayed oak wood (*Quercus petraea*, *Quercus robur*). They did not orient towards undecayed oak wood, beech (*Fagus sylvatica*), or pine wood (*Pinus sylvestris*). Although all insects tested showed anemotactic orientation, responses were nonlinear with respect to insect age. Adult females more readily oriented upwind when they were between 10 and 16 days old. Oviposition choice bioassays showed that ovipositing females would preferentially oviposit on extract-treated cellulose paper discs that had been treated with various strains of the wood-decaying fungus, *Donkioporia expansa*. HPLC-fractionated mycelial extracts were attractive to ovipositing deathwatch beetles, whereas HPLC-fractionated fungal broth extracts were repellent to ovipositing females. The implications of these results are discussed in the context of timber pest management in historically important buildings.

Key Words—Deathwatch beetle, *Xestobium rufovillosum*, Coleoptera, Anobiidae, *Quercus sp*., host selection behaviour, chemo-orientation behaviour, oviposition, timber pest, wood-decaying fungi, *Donkioporia expansa*.

* To whom correspondence should be addressed. e-mail: m.simmonds@rbgkew.org.uk
† Current address: Natural Resources Institute, University of Greenwich, Central Avenue, Chatham Maritime, Kent ME4 4TB, United Kingdom.

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INTRODUCTION

The presence of deathwatch beetles in historic buildings is reported across Europe (Fisher, 1937). It has been estimated that nearly all pre-19th century buildings containing oak timbers in England have been previously or are currently infested with deathwatch beetle (Hickin, 1963). Most evidence suggests that the beetle prefers to infest building timbers when the timber has been or is currently infested by wood-decaying fungi (Fisher, 1940; Hickin, 1974). Casual observations in buildings infested by deathwatch beetles showed that adult beetles were often found near timber-decaying fungi and that a greater number of emergence holes were found in and around fungally decayed timber (B. V. Ridout, personal communication). Unfortunately, little is known about the deathwatch beetle in its natural forest habitat. It is said that the beetle can be found in a range of tree species but that it is mainly found in the decaying parts of oak and willow trees (Fisher, 1937). The beetle is unknown to attack healthy living trees without fungal decay present (Fisher, 1940). Deathwatch beetle adults do not feed, and they live for approximately one to two months (Fisher, 1938).

The influence of decayed wood on deathwatch beetle development has been partially established (Fisher, 1940, 1941). Previous experimentation showed that deathwatch beetles demonstrated host selective oviposition preferences when given a choice between two blocks of oak timber that varied in their age (Belmain et al., 1998). Specific chemical compounds that may be important to the beetle's host selection behavior are unreported.

Generally, short-lived insects demonstrating strong host-specificity require well-developed chemoreceptors to ensure their offspring's survival (Akers and Wood, 1989). It was expected that wood-decaying fungi or decaying wood could be attractive to deathwatch beetle adults. The effects of wood-decaying fungi may be important to oviposition selection (host recognition) and/or chemoorientation (host finding). Experiments were designed to test whether walking deathwatch beetles would anemotactically orient towards airborne volatiles generated by wood-decaying fungi and timber as well as to investigate the effects of fungal extracts upon oviposition behavior.

METHODS AND MATERIALS

Adult deathwatch beetles were collected daily on emergence from a laboratory-maintained culture (21°C, 75% RH) or weekly from a deathwatch beetle-infested building (Bishopstone Church, Wiltshire, UK) at the beginning of the emergence season during April. The females used in experiments had been previously exposed to males for five days in the laboratory to allow mating to occur, but they had yet to commence ovipositing. Insects were sexed by the methods of Harris (1959) and Goulson et al. (1993).

Chemoorientation to Airborne Volatiles. The volatile odors tested were generated from pieces of European oak (*Quercus petraea*, *Quercus robur*), American oak (*Quercus rubra*), European pine (*Pinus sylvestris*), and beech wood (*Fagus sylvatica*). The wood was either of known dendrochronological age or had been decayed for standard periods of time from 4 to 20 weeks by the fungi *Fistulina hepatica*, *Coniophora putenana*, and *Donkioporia expansa* or was sound undecayed wood (Table 1). Volatiles generated from three strains of *Donkioporia expansa* grown on potato dextrose agar and *Coriolus versicolor* grown on oak wood were also tested. A blank "no odor" source was used to conduct control experiments. Odor sources were placed 30 cm away from a one-speed small domestic-use fan (fan radius = 10 cm). The amount of wood or fungus placed in front of the fan varied among experiments. Efforts were made to standardize exposed surface areas among samples; however, the amount of volatiles generated for each sample remained unknown. Smoke-generating capsules showed that air currents traveled from the fan in a straight pathway for approximately 2.5 m. Deathwatch beetles

TABLE 1. DESCRIPTION OF SAMPLES USED TO GENERATE VOLATILE ODORS IN CHEMOORIENTATION EXPERIMENTS

Volatile source	Description
Ade1	Living *Donkioporia expansa* strain FPRL 178E on potato dextrose agar
Ade2	Living *Donkioporia expansa* strain MUCL 31593 on potato dextrose agar
Ade3	Living *Donkioporia expansa* strain MUCL 30747 on potato dextrose agar
ocv20	Living *Coriolus versicolor* growing on oak wood for 20 weeks at 21°C
control	No odor source
dwb frass	Deathwatch beetle frass derived from oak wood in insect culture
ohEs	European oak heartwood sound
opEs	European oak pith sound
ohEde4	European oak heartwood incubated with *Donkioporia expansa* for 4 weeks
ohEde8	European oak heartwood incubated with *Donkioporia expansa* for 8 weeks
ohEde20	European oak heartwood incubated with *Donkioporia expansa* for 20 weeks
ohEfh10	European oak heartwood incubated with *Fistulina hepatica* for 10 weeks
ohEfh10de7	European oak heartwood incubated with *Fistulina hepatica* for 10 weeks followed by *Donkioporia expansa* for 7 weeks
ohEcp17	European oak heartwood incubated with *Coniophora puteana* for 17 weeks
ohEfh17	European oak heartwood incubated with *Fistulina hepatica* for 17 weeks
o1341	Oak tree felled in 14th century, timber obtained from a building
o1450	Oak tree felled in 15th century, timber obtained from a building
o1976	Oak tree felled in 20th century, sound untreated timber from merchant
o1986	Oak tree felled in 20th century, sound untreated timber from merchant
ohAs	American oak heartwood sound
bEcp16	European beech incubated with *Coniophora puteana* for 16 weeks
pEcp16	European pine incubated with *Coniophora puteana* for 16 weeks
pEde16	European pine incubated with *Donkioporia expansa* for 16 weeks
pEs	European pine sound

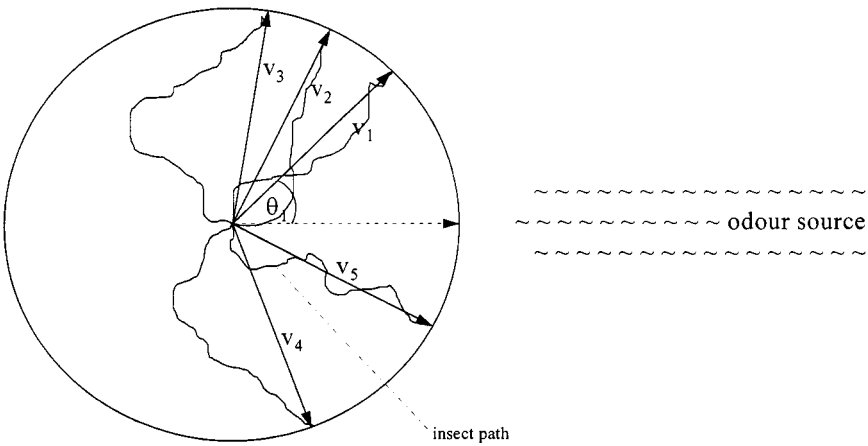


FIG. 1. Model diagram of five vectors made by the movements of five deathwatch beetles, from the center of the paper to the edge of the paper, in separate experimental trials.

were placed 1.5 m downwind from the odor source in the center of a piece of filter paper (radius = 16 cm) (Figure 1). Experiments comprised replicates of five insects of the same physiological state placed in five different standard orientations of 0°, 90°, 180°, 240°, and 300°, i.e., the insect placed at 0° would be head-first into the wind (and odor), whereas another would be rotated 180° so it was tail-first into the wind. Continual observation of each insect commenced after the individual insect was placed on the center of the paper (time zero). The insect path during the experiment was mapped, noting where the insect left the paper (end of trial). The time to complete the experiment was recorded. Trials where the insect had not left the paper after 60 min were aborted. All laboratory experiments were conducted under even lighting conditions at 21 ± 1°C and 35 ± 5% RH. Insects from Bishopstone Church were tested after five days in the laboratory against 24 odor sources (Table 1). Adult females from the laboratory culture were tested at known times after their emergence against four odor sources (o1341, ocv20, dwb frass, control).

The insect response to a volatile is represented by a vector. This vector was created by drawing a straight line from the center of the paper (the point where the insect was placed at the start of the experiment) to the point at the edge of the paper where the beetle departed (Figure 1). Therefore, each vector is of the same magnitude, i.e., the radius of the filter paper (16 cm), whereas the angle between the vector and the odor source varied.

Oviposition Preference Bioassay. Different strains of the wood-decaying fungus, *Donkioporia expansa*, were obtained from Belgium (MUCL), The Netherlands (CBS), and Great Britain (FPRL) (Table 2). Fungi were grown in liquid media, and

TABLE 2. DESCRIPTION OF FUNGAL EXTRACT SAMPLES USED IN HOST SELECTION EXPERIMENTS TESTING FOR OVIPOSITION PREFERENCE USING A CHOICE TEST BIOASSAY

Extract source	Fungal extract sample
5hm2f	5-Hydroxymethyl-2-furancarboxaldehyde
CBS299.93bH2	CBS 299.93 broth *n*-hexane fraction 2
D23691e	*Donkioporia* 23691 exudate
D31865e	*Donkioporia* 31865 exudate
DridoutIIe	*Donkioporia* ridout II exudate
Ergtype	Ergosterol type compound
FPRL178Ee	FPRL 178E exudate
FPRL178EmE1	FPRL 178E mycelium EtOAc fraction 1
MUCL30747bE1	MUCL 30747 broth EtOAc fraction 1
MUCL30747bE2	MUCL 30747 broth EtOAc fraction 2
MUCL30747e	MUCL 30747 exudate
MUCL30747mE3	MUCL 30747 mycelium EtOAc fraction 3
MUCL30747mH1	MUCL 30747 mycelium *n*-hexane fraction 1
MUCL31593mE1	MUCL 31593 mycelium EtOAc fraction 1
MUCL31593mE2	MUCL 31593 mycelium EtOAc fraction 2
MUCL31593mE3	MUCL 31593 mycelium EtOAc fraction 3
tri2hb	Trisubstituted 2-hydroxybenzaldehyde

extracts of mycelial and broth fractions in hexane and ethylacetate were fractionated by HPLC by collaborators in Dublin, Republic of Ireland (D. M. X. Donnelly and I. Nesbitt). Fractions were dried, weighed and diluted in hexane to a concentration of 1000 ppm. Fungal exudates that were collected from different fungal strains and fungally derived purified compounds were similarly tested at 1000 ppm.

An aliquot (100 μl) of a extract was applied to cellulose paper discs (2 cm diameter) (Whatman) and allowed to dry. The discs treated with the extract were placed individually in a Petri dish (8.5 cm diameter) along with a solvent only (hexane) control disc. One mated female deathwatch beetle was placed in each Petri dish, and the number of eggs oviposited on the discs was counted daily until insect death ($N = 10$). The experimental arenas were kept in a dark quiet room at $21 \pm 1^{\circ}$C and $35 \pm 5\%$ RH. Participation in the experiment was determined by the oviposition of at least one egg.

RESULTS

Chemoorientation to Airborne Volatiles. Responses of adult females to volatiles varied according to their age and the source of the volatile. For example, the mean time taken for the insects to move from the center of the paper to the edge of the paper was shown to be less for samples of decayed oak (ohEde4, ohEde8) and living fungus (ocv20, Ade1, Ade2, Ade3) than for samples of European pine

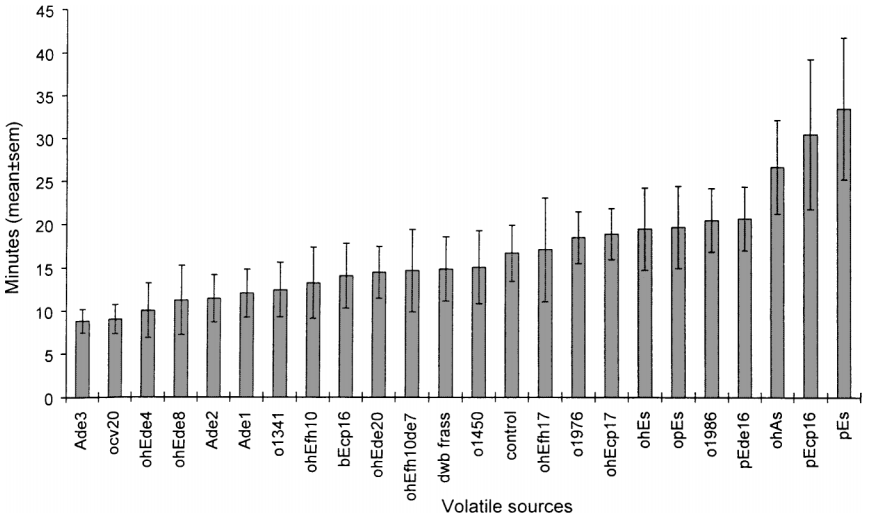


FIG. 2. Time (minutes) taken for deathwatch beetle mated females (collected from Bishopstone Church) to complete the chemoorientation experiment by leaving the edge of the filter paper when originally placed in the center of the disc ($N = 5$).

(pEs, pEcp16) and undecayed oak (ohAs) (Mann-Whitney U tests, $P < 0.05$; Figure 2). The effects of insect age on beetle response to three odors (o1341, ocv20, and dwb frass) showed no difference in the mean time taken to complete experiments among the insect ages tested (Mann-Whitney U tests, $P > 0.05$).

The mean vector angle for an experiment ($N = 5$) was shown to vary among experiments according to the source of the volatile odor and insect age. The mean angle was smaller (20°–40°), that is, the insect moved more directly towards the source when the odor source was from living fungi (ocv20, Ade1, Ade2, Ade3), old oak (o1341, o1450), or decayed oak wood (ohEfh10de7, ohEfh10, ohEcp17, ohEde20, ohEfh17) when compared with the mean angle (55°–110°) from modern oak (o1976, o1986), undecayed oak (ohAs, ohEs, opEs), beech (bEcp16), or pine (pEs, pEde16, pEcp16) (Mann-Whitney U tests, $P < 0.05$) (Figure 3). The mean vector angle varied with insect age when the volatile odor source was from o1341 (Figure 4) and ocv20 (Figure 5) (Mann-Whitney U tests and Kruskal-Wallis, $P < 0.05$). These two odor sources showed that the mean vector angle initially decreased with insect age until insects were approximately 11 days old and that the mean vector angle increased with insect age after insects were older than 14 days. Insect age had no effect on the mean vector angle when the odor source was generated from deathwatch beetle frass (dwb frass) (Figure 6) or in the control (no odor) experiment (Mann-Whitney U tests and Kruskal-Wallis, $P > 0.05$).

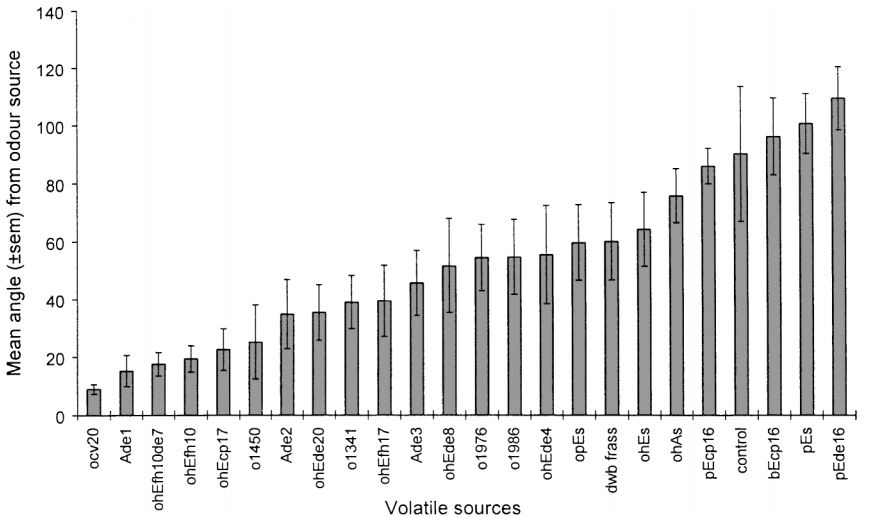


FIG. 3. Mean vector angle of vectors generated by wild mated female deathwatch beetles exposed to airborne volatiles in chemoorientation experiments ($N = 5$).

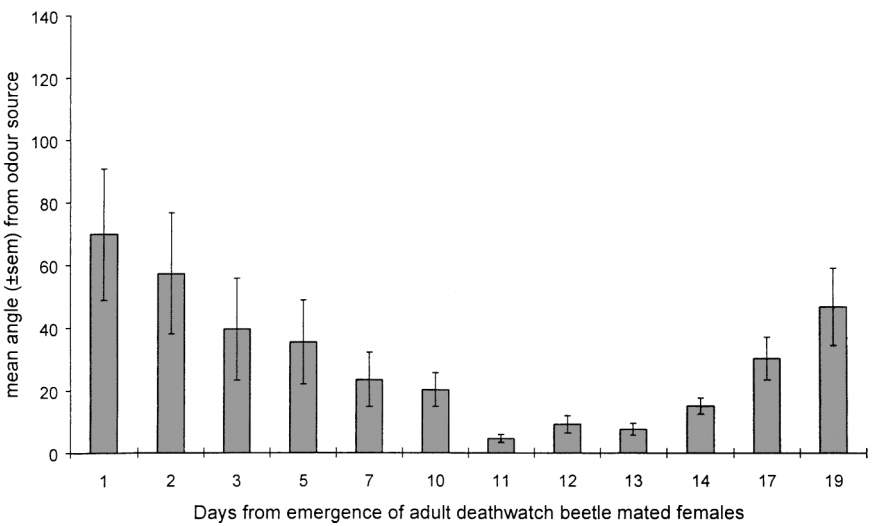


FIG. 4. Mean vector angle of vectors generated by mated female deathwatch beetles that emerged from a laboratory culture. Insects of known age were exposed to airborne volatiles from oak wood cut down in the year 1341 ($N = 5$).

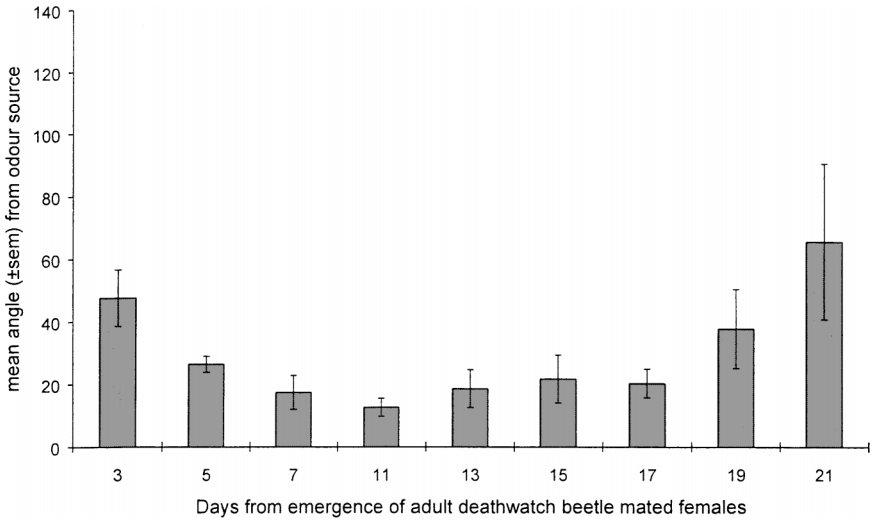


FIG. 5. Mean vector angle of vectors generated by mated female deathwatch beetles that emerged from a laboratory culture. Insects of known age were exposed to airborne volatiles from oak wood infected with *C. versicolor* ($N = 5$).

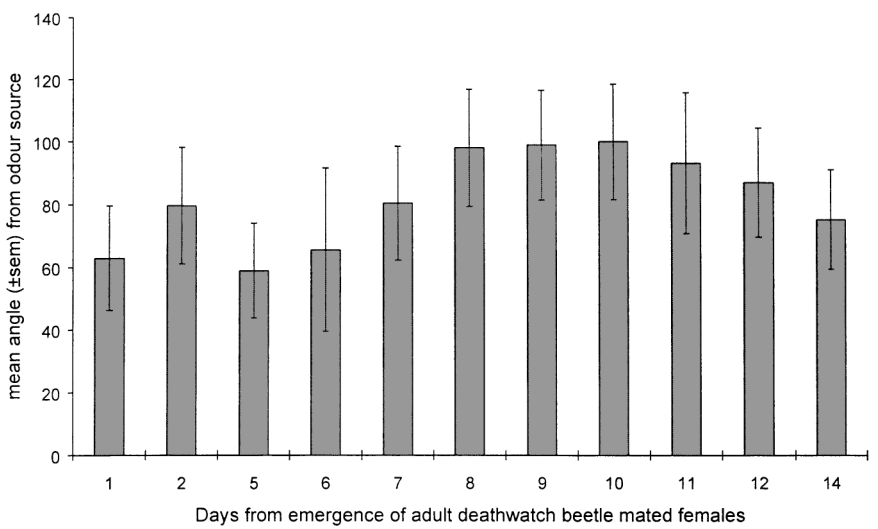


FIG. 6. Mean vector angle of vectors generated by mated female deathwatch beetles that emerged from a laboratory culture. Insects of known age were exposed to airborne volatiles of deathwatch beetle frass from laboratory insect culture wood ($N = 5$).

Variance of the mean vector angle was shown to indicate strength of insect response. High variance, as in the control experiment, indicated a lack of a directed response to the odor source as insects did not uniformly orient upwind, downwind, or across wind, i.e., insects did not orient to the wind and left the paper in the direction of their standard angle of orientation. High variance and high mean angle, therefore, indicated there was no insect response to a volatile. Low variance of the mean vector angle showed that odor sources were either attractive (when the mean angle was small) or repellent (when the mean angle was high). Linear and nonlinear regression models showed no correlation between variance and mean angle ($r^2 < 0.35,\ P = 0.05$). Variance among experiments that showed a low mean vector angle (0°–40°) was less than the variance among experiments that showed a high mean vector angle (55°–110°) (F tests at $P = 0.05$), indicating that insects demonstrated a less variable response to attractive odors than unattractive odors.

Oviposition Preference Bioassay. Some fungal extract-treated discs were preferentially oviposited upon, when compared to the control (no treatment) disc, whereas other extract-treated discs were avoided with insects ovipositing all their eggs on the control disc (Table 3, Figure 7). In some experiments, the extract

TABLE 3. RESULTS OF OVIPOSITION CHOICE BIOASSAYS WHERE FEMALES ARE PRESENTED WITH FUNGAL EXTRACT-TREATED CELLULOSE DISCS[a]

Extract	Response	Test value	P
MUCL 31593 mycelium EtOAc fraction 3	attractive	2.1405	0.0323
MUCL 31593 mycelium EtOAc fraction 1	attractive	2.3953	0.0166
5-Hydroxymethyl-2-furancarboxaldehyde	attractive	2.6502	0.0080
MUCL 31593 mycelium EtOAc fraction 2	attractive	2.6656	0.0077
FPRL 178E mycelium EtOAc fraction 1	attractive	2.8031	0.0051
MUCL 30747 mycelium EtOAc fraction 3	attractive	2.8031	0.0051
MUCL 30747 mycelium *n*-hexane fraction 1	attractive	2.8031	0.0051
Control	no discrimination	0.1185	0.9057
Trisubstituted 2-hydroxybenzaldehyde	no discrimination	0.1185	0.9057
MUCL 30747 exudate	no discrimination	0.4193	0.6750
FPRL 178E exudate	no discrimination	0.5916	0.5541
Donkioporia 23691 exudate	no discrimination	0.9435	0.3454
Donkioporia ridout II exudate	no discrimination	1.4832	0.1380
Donkioporia 31865 exudate	repellent	2.0226	0.0431
Ergosterol type compound	repellent	2.0896	0.0367
MUCL 30747 broth EtOAc fraction 2	repellent	2.6502	0.0080
MUCL 30747 broth EtOAc fraction 1	repellent	2.8031	0.0051
CBS 299.93 broth *n*-Hexane fraction 2	repellent	2.8031	0.0051

[a] 1000 ppm, Wilcoxon matched-pairs signed-ranks test on the mean number of eggs per disc, $N = 10$. An attractive response was when eggs were preferentially laid on the treated disc. A repellent response was when eggs were preferentially laid on the untreated control disc. No discrimination was when a significant preference could not be determined.

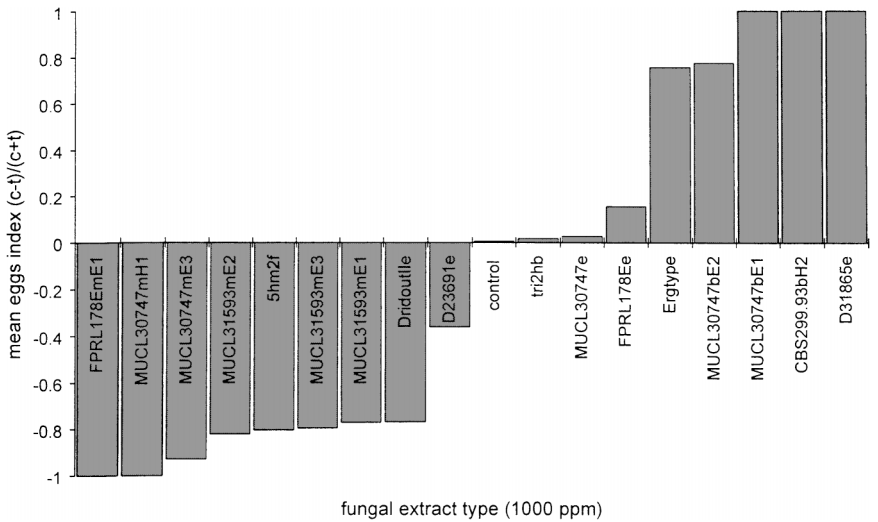


FIG. 7. Oviposition preference choice test bioassays ($N = 10$) using extracts of the wood decaying fungus *Donkioporia expansa* on cellulose discs showing an oviposition index that compares the mean number of eggs oviposited on control discs as opposed to treated discs; -1 shows all eggs were oviposited on the treated disc and $+1$ shows all eggs were oviposited on the control disc.

did not influence oviposition behavior as eggs were laid on treatment and control discs in a similar fashion to that when two control discs were present. Generally, mycelial fractions were found to be attractive, whereas broth fractions were found to be repellent when tested at the same concentration (Table 3). Fungal exudates appeared to have no significant effect on oviposition behavior.

In order to compare among experiments the variable oviposition response to the extracts, indices were calculated by the formula $(C - T)/(C + T) =$ oviposition index, where C and T represent the number of eggs laid on the control and treatment discs, respectively. Similar results were shown among experiments in the relative magnitude of oviposition response to the extract when oviposition indices were calculated from the mean number of eggs laid on the control and treatment discs (Figure 7).

DISCUSSION

The chemoorientation behavior of female deathwatch beetles would suggest that walking adult deathwatch beetles can orient upwind to potential host volatiles (Hansell, 1985; Visser, 1988). The observed differences in the strength of response

to various volatile sources could have been due to the particular volatiles associated with the source samples, or it could have been due to the amount of volatile produced or both (Heinzel and Böhm, 1984; Visser and Thiery, 1986; Akers and Wood, 1989). The similarity of insect response among odor sources of a similar nature would argue that the volatile composition was very important to the overall chemoorientation behavior demonstrated. For example, decayed oak samples and living fungal samples were attractive despite potential differences in volatile concentration or composition among odor sources. Similarly, undecayed oak and pine odors did not elicit an upwind orientation response. It is unknown what particular compounds or ratio of compounds were important for this chemoorientation behavior, but there may be combinations of compounds that could cause chemoorientation, as several different volatile sources demonstrated similar insect orientation behaviors (Visser, 1983; Miller and Strickler, 1984).

Volatiles from decayed oak and living fungi showed that insects readily walked upwind, regardless of their initial physical orientation to the wind at the commencement of the experiment, and variability among insect responses was relatively low as opposed to the control (no odor). Pine wood and undecayed wood produced a less definite insect response in that beetles tended to walk across the wind, downwind, or behaved similarly to those in the control experiment where insects walked off the paper in the direction of their initial orientation to the wind. Therefore, the experiment was inconclusive in determining whether some odors were repellent or simply unattractive (Heinzel and Böhm, 1984; Preiss and Kramer, 1986). However, variance of the mean angle was higher among experiments with a large angle from the odor source as opposed to experiments with a low angle from the odor source. This relative increase in variability among experiments of high angle suggests that some insect responses (low mean variance) were caused by repellent odors, whereas some insect responses (high mean variance) may indicate insects did not react to the odor. Choice-preference experiments using choice olfactometers (Ponsonby and Copland, 1995; Potting et al., 1995; Steinberg et al., 1992) could perhaps define a repellent volatile mix as opposed to a volatile mix that elicits no response.

Adult females appeared to have an optimal period when they were most responsive to volatile odors. Although insects of all ages did orient to attractive volatiles, the response was more directed when insects were at the age when oviposition usually occurred (10–16 days old). This could argue that, to some extent, chemoorientation behavior is physiologically driven as responsiveness to volatiles from the same odor source varied with insect age (Bell et al., 1995).

It was already known that deathwatch beetles demonstrate oviposition preferences when given a choice between two blocks of timber (Belmain et al., 1998). The oviposition bioassays using fungal extracts additionally showed that deathwatch beetles can at least partially select oviposition sites by contact chemoreception of fungally derived compounds. Fungally decayed oak contains decomposition

products, such as methylfuroate and dimethoxybenzene, as well as some uncharacterized furan compounds (Esser and Tas, 2001). Further experiments are required to assess whether the deathwatch beetles respond to any of these compounds. Concentration and the relative ratios of compounds in extracts could affect the overall oviposition response (Ridgway et al., 1990).

Many insects have demonstrated both host selection behavior from a distance (host plant finding) and on contact with the surface of the host (host plant recognition) (Bell et al., 1995). Chemoorientation has been argued to be a common insect trait as primitive insects such as cockroaches demonstrated both temporal and spatial processing of olfactory information (Bell and Kramer, 1979). It should, therefore, not be surprising that the deathwatch beetle is also capable of host selection using chemoorientation and oviposition selection to determine its optimal host in order to increase offspring fitness. The identification of beetle-attracting compounds could lead to improved pest control in deathwatch beetle-infested buildings through trap-baiting (Day and Sjogren, 1994) or monitoring as part of an integrated pest management (IPM) strategy (Simmonds et al., 2001). Research on the identification of chemoreceptors on the insect and subsequent electrophysiological experiments (Blaney et al., 1987; Blaney and Simmonds, 1990) would help confirm host selection behavior of the deathwatch beetle.

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ACTIVATION OF SOIL RESPIRATION AND SHIFT OF THE MICROBIAL POPULATION BALANCE IN SOIL AS A RESPONSE TO *Lavandula stoechas* ESSENTIAL OIL

D. VOKOU,[1,*] D. CHALKOS,[1] G. KARAMANLIDOU,[2] and M. YIANGOU[2]

[1]*Department of Ecology*
[2]*Department of Genetics, Molecular Biology and Development*
School of Biology
Aristotle University
54 006 Thessaloniki, Greece

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Abstract—*Lavandula stoechas*, a native plant of Greece, is rich in essential oil and fenchone is its major constituent. We examined the effect of the essential oil and its main constituents on soil metabolism and microbial growth. Addition of the essential oil or fenchone to soil samples induced a remarkable increase in soil respiration. This was accompanied by an increase in the soil bacterial population of three orders of magnitude. This sizable population was not qualitatively similar to that of the control soil samples. One bacterial strain dominated soil samples treated with *L. stoechas* essential oil or fenchone. By use of the disk diffusion assay, we evaluated the capacity of three bacterial strains that we isolated from the soil samples, as well as *Escherichia coli* and *Bacillus subtilis* (reference strains), to grow in the presence of the essential oil and three of its main constituents (fenchone, cineol, α-pinene). The substances tested did not inhibit the growth of the strain found to dominate the bacterial populations of treated soil samples; they severely inhibited *B. subtilis*. The other two isolated strains could also grow in liquid cultures in the presence of different quantities of essential oil or fenchone. Addition of fenchone at the end of the exponential phase increased the cell numbers of the strain that dominated the bacterial populations of treated soil samples, indicating use of the substrate added. On the basis of these results, we propose a scheme of successional stages during the decomposition process of the rich-in-essential-oil litter of aromatic plants that abound in the Mediterranean environment.

Key Words—*Lavandula stoechas*, soil metabolism, microbial growth, essential oil.

*To whom correspondence should be addressed. E-mail: vokou@bio.auth.gr

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INTRODUCTION

Aromatic plants make a remarkable contribution to the flora and vegetation of the Mediterranean environment. Common among them are representatives of the family Lamiaceae (Kokkini et al., 1989). A distinctive feature of this family of plants is their production of essential oils, which are mixtures of low-molecular-weight isoprenoids. The antimicrobial activity of these compounds is widely known (Janssen et al., 1987; Bennett et al., 1994; Sivropoulou et al., 1995, 1996, 1997; Amaral and Knowles, 1998; Karamanoli et al., 2000).

Despite the fact that essential oils characterize the fragrant Mediterranean environment, their function is not known. In this environment, decomposition is limited by unfavorable combinations of temperature and rainfall (Vokou, 1992). If the antimicrobial activity of essential oils held for soil microorganisms as well, decomposition would be critically affected, as biological constraints (essential oils inhibiting microorganisms) would function on top of physical ones. Therefore, our goal has been to understand how essential oils, known to possess antimicrobial activity, affect soil microorganisms and, concomitantly, the decomposition process.

Results of our previous research showed that soil respiration was activated in the presence of *Coridothymus capitatus* and *Satureja thymbra* essential oils. The increase of soil respiration was found to be a primary rather than secondary effect. The essential oils from these plants did not kill some soil microorganisms, thereby providing substrate easily decomposable to others (Vokou et al., 1984) but directly activated soil bacteria.

We further examined the effect of a number of individual essentail oil constituents on soil respiration and found that the compounds tested (oxygenated or not) resulted in increased CO_2 release from soil samples collected from the area where these aromatic plants grow. The observed differences were mostly of a temporal nature. Some were immediately effective, while others required a time lag (Vokou and Margaris, 1988). Given these responses, we examined the effect of essential oils of largely different chemical composition from both indigenous and nonindigenous aromatic plants on soil samples of different origin. The essential oils tested were rich in phenols, carvacrol, and/or thymol (from *Origanum vulgare* subsp. *vulgare*, *C. capitatus*, and *S. thymbra*), in carvone–dihydrocarvone (*Mentha spicata*), in 1,8-cineol–camphor (*Rosmarinus officinalis*), and in linalool–linalyl acetate (*Lavandula angustifolia*, an introduced species). The soil samples were collected from beneath wild growing aromatic plants, from open spaces without a shrub layer, and from areas not exposed to essential oils (e.g., from cultivated fields). In spite of their different chemical composition, all of the essential oils activated soil respiration. Moreover, soils not previously exposed to essential oils responded similarly to those supporting aromatic plants (Vokou and Liotiri, 1999). These studies suggest that the antimicrobial activity of essential oils is not

expressed against all soil microorganisms. Some can metabolize them and use them as carbon and energy sources.

The ability of some bacteria to use volatile organic compounds (VOC), including essential oils, has been well documented. They can degrade monoterpenes, such as α- and β-pinene, carene, limonene, γ-terpinene, and *p*-cymene (Dhavalikar and Bhattacharya, 1966; Gibbon and Pirt, 1971; Griffiths et al., 1987; Stumpf et al., 1990; Harder and Probian, 1995; Misra et al., 1996; Harms et al., 1999; Kleinheinz et al., 1999) or oxygenated compounds, such as camphor (Gunsalus and Marshall, 1971). The bacteria are both Gram-negative (e.g., *Pseudomonas*, *Alcaligenes*) and Gram-positive (e.g., *Mycobacterium*, *Nocardia*). As stated by Misra and Pavlostathis (1997), although monoterpenes are easily degraded in liquid as well as soil–slurry systems under aerobic conditions, "much remains to be studied for a more complete understanding of the behavior of monoterpenes in the natural environment."

The aims of the present work are (1) to provide additional experimental evidence in support of our hypothesis that essential oils increase always soil respiration, and (2) to examine how changes in the soil microbial population shift toward microorganisms resistant to essential oils, able to use them as carbon and energy sources, and facilitate the decomposition process of aromatic plant litter. Towards these aims *Lavandula stoechas* (Lamiaceae) was used. This plant is native in Greece and has a wide occurrence across the country. Its essential oil is dominated by the oxygenated compound fenchone. Neither the essential oil nor fenchone, its major constituent, have been studied for their effect on soil microorganisms.

METHODS AND MATERIALS

Essential Oil and Constituents. Plant material of *L. stoechas* was collected in the wild from Halkidiki, N. Greece. Leaves were separated and water distilled in a Clevenger apparatus for 2.30 hr. The essential oil extracted from leaves was analyzed by gas chromatography (Varian 3700) by using previously described conditions (Vokou et al., 1993). Major constituents were identified on the basis of retention times and by cochromatography of authentic samples.

The crude essential oil and its constituents fenchone, cineol, and α-pinene (Aldrich Chemical Co., Milwaukee, Wisconsin) were used in the experiments.

Soil Respiration Studies. Soil samples were collected from the upper soil layer ($<$5 cm) in an area with Mediterranean vegetation, close to Thessaloniki University campus. The soil was air-dried and sieved ($<$1 mm). Soil respiration was determined by using the method described in Vokou and Liotiri (1999). In brief, soil samples (150 g dry wt) were put in airtight containers (aluminum cylinders with approximately 1 liter capacity), 30 ml of distilled water added to the soil samples, and a 50-ml beaker with 20 ml 1M KOH was placed on the top of the

soil in each container. Respiration was estimated under the following conditions: (1) from soils treated with *L. stoechas* essential oil, (2) from soils treated with fenchone (major constituent of the essential oil), (3) from untreated soils, and (4) from a control without soil but containing the beaker with KOH. In the treated soils, 0.1 ml of either essential oil or fenchone was added. Three replicates were used for each treatment. Experiments were conducted at room temperature (20–27°C). CO_2 absorbed by KOH was estimated by titration with 0.1 ml HCl after seven days based on previous studies (Vokou et al., 1984; Vokou and Margaris, 1988; Vokou and Liotiri, 1999). The essential oil or fenchone was added repeatedly in the same soil samples at the same quantity, on a weekly basis.

Counts, Isolation, and Identification of Bacterial Strains. We examined soil samples treated with essential oil, soil samples treated with fenchone, and control soil samples. The soil dilution plate method on Oxoid nutrient agar was used, and the number of bacterial colonies was counted. Three replicates were used for each treatment.

Pure cultures of bacterial strains were obtained. Isolated bacterial strains were examined for colony morphology (visual), cell morphology (light microscope), Gram reaction, and response to biochemical tests. Biochemical tests for each isolated strain were repeated twice and gave identical results. For bacterial identification, the commercially supplied systems MICROBACT 12A (for strains negative to oxidase) and MICROBACT 12B (for strains either positive or negative to oxidase) were used, as well as Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Bacterial counts and bacterial composition of untreated soils and those treated with *L. stoechas* essential oil and fenchone were compared.

Disk Diffusion Assay. The tolerance against *L. stoechas* essential oil and its constituents, fenchone, cineol, and α-pinene, of the three isolated bacterial strains from soil samples and of *Bacillus subtilis* (NCIMB 3610) and *Escherichia coli* (NCIMB 8879), purchased from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, Scotland), were tested. The latter two bacteria are standard reference strains routinely used for the evaluation of antimicrobial compounds and shown to be sensitive to various essential oils (Sivropoulou et al., 1995).

The essential oil and each constituent were examined at four different quantities: 1, 5, 10, and 15 μl. Filter paper disks (Whatman No. 1, 5 mm diameter), containing the essential oil or individual constituents were applied to the surface of agar plates previously seeded with 0.2 ml of the test organisms. There were four replicates for each treatment. Plates were incubated overnight at 30°C. The diameters of the resulting zones of inhibition were measured in millimeters. The net zones of inhibition were calculated after subtraction of the diameter (5 mm) of the paper disk.

Bacterial Cell Growth. Well-isolated, single bacterial colonies were grown overnight at 37°C in nutrient broth. Two sets of experiments were done to monitor

bacterial growth. In the first set, the growth of two bacterial strains isolated from the soil and *E. coli* were followed in presence of *L. stoechas* essential oil or fenchone, added at quantities of 0.1, 0.25 and 0.5 ml in tubes of nutrient broth (150 ml); control tubes for each strain were also used. The nutrient broth was inoculated with appropriate aliquots of the cultures of these bacteria so that their optical densities (OD_{600}) were similar. We monitored the growth of each culture by measuring optical density (OD_{600}) at 30-min intervals for 8 hrs. In the second set, the potential of the same two soil bacterial isolates and *E. coli* to grow in nutrient broth enriched with fenchone added at the end of the exponential phase of their growth was monitored. Fenchone was tested at 0.2 and 0.5 μl/ml and bacterial growth was monitored as above. Experiments were repeated twice and yielded essentially the same results.

Statistical Analysis. SPSS was used for statistical analyses. To estimate whether the effect of treatments was significant, analysis of variance was conducted followed by multiple LSD comparisons; *t* test was run in case of two-sample comparisons.

RESULTS

L. stoechas is a rich-in-essential-oil aromatic plant; the yield from its leaves was 2.3 ml/100 g dry wt. The chemical composition of its essential oil was found to be similar to that previously described for the species from a population at Halkidiki (Kokkalou 1987), from where we also took our samples. The main constituents were fenchone (41%), cineol (29%), and α-pinene (1.6%).

Addition of *L. stoechas* essential oil or fenchone increased the CO_2 released from treated soil samples compared to the control ($P < 0.001$) (Figure 1); soil respiration was maintained at high levels with repeated additions. There was no significant difference in soil respiration between the effect of *L. stoechas* essential oil and fenchone.

Bacterial counts from control soil samples and those treated with *L. stoechas* essential oil or fenchone are consistent with the observed increase in soil respiration. The counts rose by approximately three orders of magnitude in the presence of *L. stoechas* essential oil or fenchone (Table 1). The difference between bacterial counts from treated soil samples and the control was significant ($P < 0.001$).

Three Gram-negative bacterial strains were isolated and tentatively identified from treated soils (Table 2) including *Aeromonas hydrophila* (strain I), *Pseudomonas putida* (strain II), and *Vibrio alginolyticus* (strain III). However, we consider these identifications as tentative (particularly those for *A. hydrophila* and *V. alginolyticus*) because the substrate preferences do not fit well with the substrates that we used. For this reason, we will continue to refer to the isolates as strains I, II, and III.

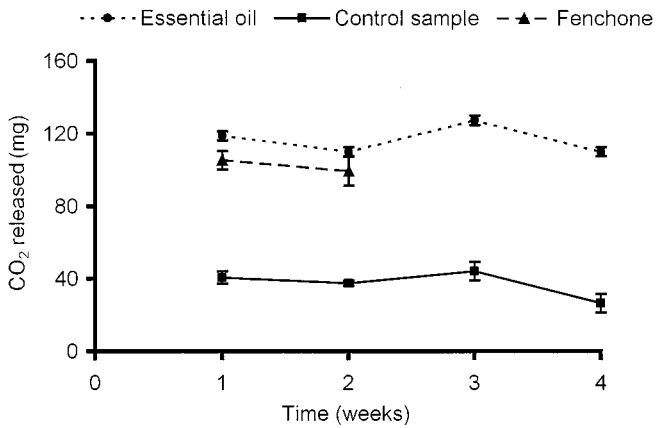


FIG. 1. Soil respiration in control samples and those treated with 0.1 ml of *L. stoechas* essential oil or fenchone. Values represent CO_2 released per week and were taken one week after *L. stoechas* essential oil or fenchone was added to soil samples (150 g dry wt).

Strain I was the most abundant in soils treated either with *L. stoechas* essential oil or fenchone. In fact, strain I accounted for 95% of the microbial population in soil treated with essential oil, for 78% in soil treated with fenchone, but only 13% in control soil (Table 1). Criteria used to assign bacterial counts to this strain included visual (morphology of the colony) and microscopic (light microscope); assignment was facilitated by the greenish color of strain I colonies that clearly differentiated it from all other strains.

The ability of the three isolated bacterial strains and two reference strains, *Escherichia coli* and *Bacillus subtilis*, to grow in the presence of *L. stoechas* essential oil (Figure 2a), the oxygenated monoterpenoids fenchone (Figure 2b) and cineol (Figure 2c), and the nonoxygenated monoterpene α-pinene (Figure 2d) was evaluated. None of the compounds affected the growth of strain I. The essential oil and the two oxygenated compounds, at quantities ≥ 5 μl, had a weak effect on

TABLE 1. SIZE OF SOIL BACTERIAL POPULATION AND PERCENT PARTICIPATION OF STRAIN I IN CONTROL SOIL SAMPLES AND THOSE TREATED WITH *L. stoechas* ESSENTIAL OIL OR FENCHONE

Sample	Bacterial population (CFU/g)	Participation of strain I (%)
Control	$3.9 \pm 0.2 \times 10^6$	13
Treated with *L. stoechas* essential oil	$3.1 \pm 0.3 \times 10^9$	95
Treated with fenchone	$2.6 \pm 0.2 \times 10^9$	78

TABLE 2. MORPHOLOGY AND RESPONSE TO BIOCHEMICAL TESTS OF BACTERIAL STRAINS ISOLATED FROM TREATED SOIL SAMPLES

Morphology and reactions to biochemical tests	Strain I	Strain II	Strain III
Shape	rod	rod	rod
Gram reaction	−	−	−
Motility	+	+	−
Nitrates	−	−	+
Oxidase	+	+	+
Catalase	+	−	−
Amylase	−	−	−
Lysine	+	+	+
Ornithine	+	+	+
Hydrogen sulfide	−	−	−
Glucose	+	−	−
Manitol	−	−	−
Xylose	−	−	−
Orthonitrophenol galactopyranoside (ONPG)	−	+	−
Indole	+	+	+
Urease	+	+	−
Voges-Proskauer (VP)	+	−	+
Citrates	+	+	−
Tryptophane deaminase (TDA)	−	−	−
Gelatin	+	−	−
Malonate	−	−	−
Inositol	−	−	−
Sorbitol	−	−	−
Ramnose	−	−	−
Sucrose	−	−	−
Lactose	−	−	−
Arabinose	+	−	−
Adonitol	−	−	−
Raffinose	−	−	−
Salicin	−	−	−
Arginine	+	+	+
Bacterial strain	*Aeromonas hydrophila*	*Pseudomonas putida*	*Vibrio alginolyticus*
Percent probalility after MICROBACT system	88.5	81.3	98.7

the growth of strain II (maximum inhibitory zone, 5 mm), whereas α-pinene was inactive. Their effect on strain III was similar; maximum inhibition was recorded in presence of 15 μl of the essential oil. *E. coli* responded similarly to strains II and III in most treatments, but fenchone and cineol considerably inhibited its growth at the maximum quantity tested (15 μl). *B. subtilis* was the most sensitive strain. The

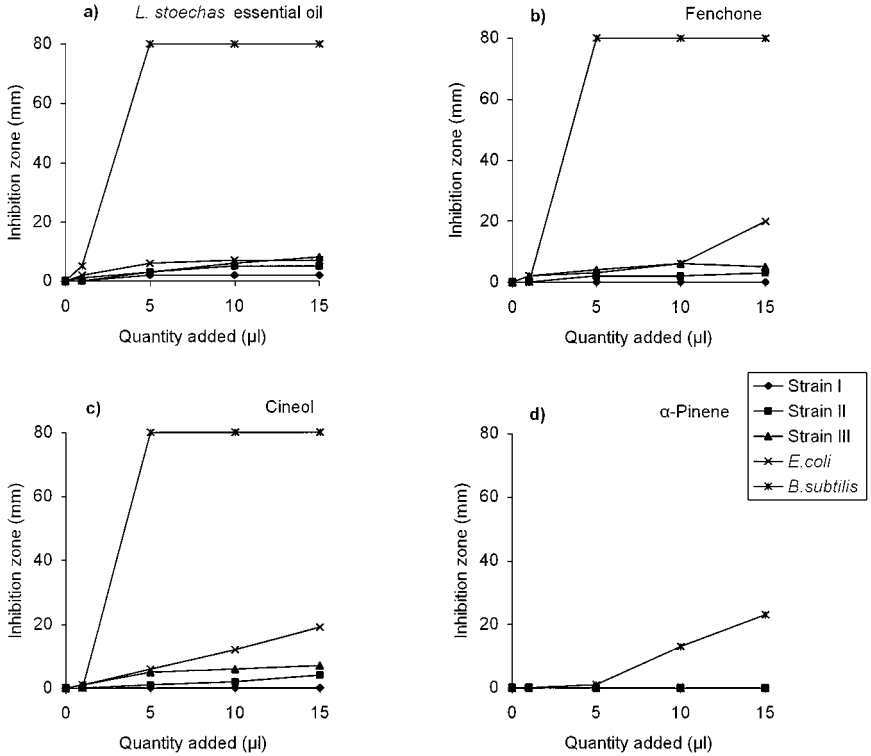


FIG. 2. Antimicrobial activity of (a) *L. stoechas* essential oil, (b) fenchone, (c) cineole, and (d) α-pinene, applied at four different quantities, against strains I, II, and III, *E. coli*, and *B. subtilis.*

essential oil, fenchone, and cineol entirely inhibited its growth at quantities ≥ 5 μl. α-Pinene, which was inactive against all other bacterial strains, was inhibitory at quantities ≥ 10 μl. Two-way ANOVA was run on data taken after addition of the maximum quantity (15 μl) with bacterial strains and compounds used as factors; both bacterial strains and compounds had significant effects. Multiple LSD comparisons showed that all differences in inhibition among species were significant. Differences among compounds were significant except between fenchone and cineol, the two oxygenated compounds.

The liquid-phase assays, designed to test a compound's activity when added either at the beginning or the end of the exponential phase of microbial growth, relied on the results of the disk diffusion assays to choose both the compound and the bacterial strains to be tested. Fenchone was chosen as the most inhibitory of the three compounds tested; strains I and II were selected as the most resistant

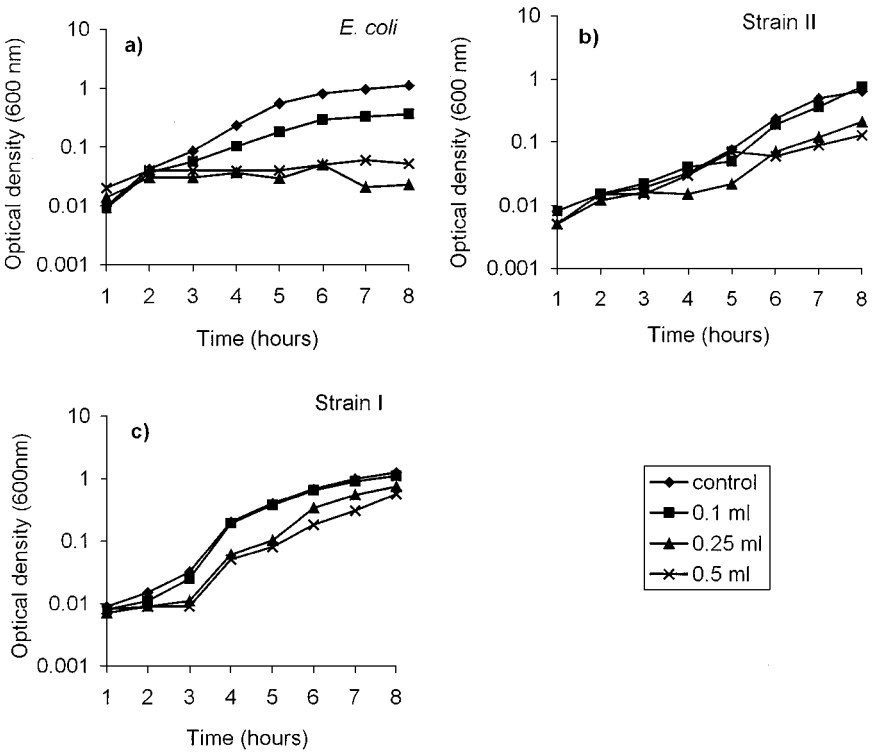


FIG. 3. Time- and concentration-dependent effect of fenchone on the growth rate of *E. coli* (a), strain II (b), and strain I (c). Quantities of fenchone were added to 150 ml of culture medium. Experiments were repeated twice. Each value in the graphs is the average of two; individual values differed by less than 3% from the mean.

soil bacterial strains. For the sake of comparison, the essential oil and *E. coli* were also examined. Results of the assay with fenchone and *L. stoechas* essential oil, added at the beginning of the exponential phase are given in Figures 3a–c and 4a–c, respectively. At the end of the experimental period (8 hr), strains I and II continued to grow in the presence of fenchone (Figure 3b,c), and growth curves were similar to controls. This was not the case for *E. coli*, which was substantially inhibited in presence of 0.25 and 0.5 ml of fenchone (Figure 3a). The growth of *E. coli* in presence of the essential oil was similar (Figure 4a). Strains I and II grew under all quantities examined, but strain I at a lower rate in the case of the highest quantities (Figure 4b,c).

When added at the end of the exponential phase of microbial growth, strain I continued to grow in the presence of fenchone, in contrast to strain II and *E. coli* (Figure 5). When a microbial population reaches the stationary phase of its growth,

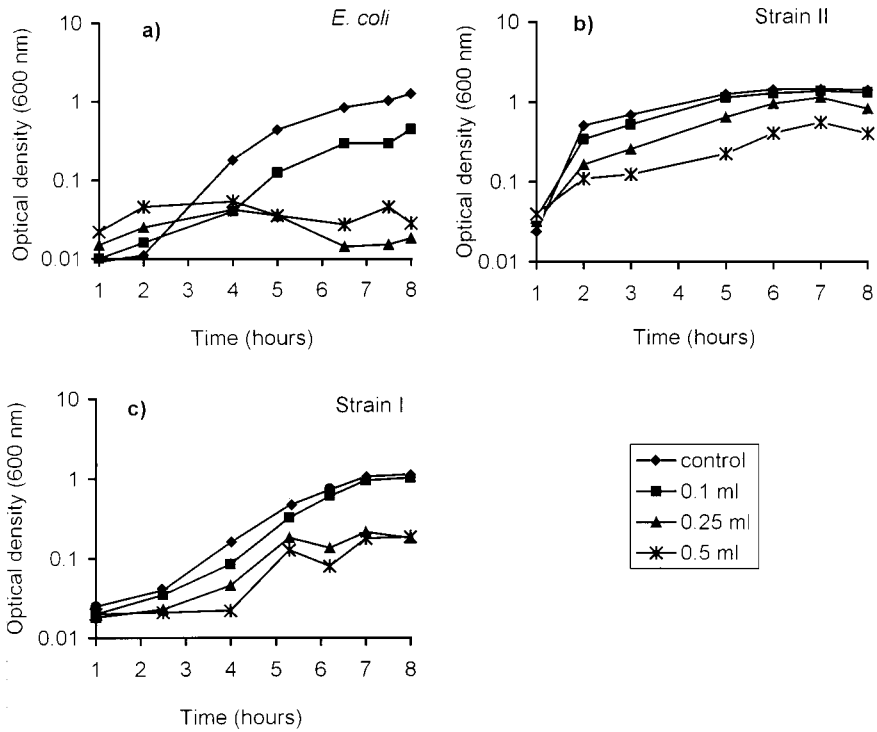


FIG. 4. Time- and concentration-dependent effect of *L. stoechas* essential oil on the growth rate of *E. coli* (a), strain II (b), and strain I (c). Quantities of essential oil were added to 150 ml of culture medium. Experiments were repeated twice. Each value in the graphs is the average of two; individual values differed by less than 3% from the mean.

it means that it has consumed all carbon and energy sources of the culture medium. Further increases in population size, after a substrate (fenchone, in this case) is added, indicates use of this substrate.

DISCUSSION

Addition of *L. stoechas* essential oil to soil samples induced a remarkable increase in soil respiration. Fenchone induced a similar response. These results are consistent with other studies using different essential oils (Vokou et al., 1984; Vokou and Liotiri, 1999) or individual compounds (Vokou and Margaris, 1988) and support the contention that essential oils, irrespective of their chemical composition, have the potential to increase soil respiration.

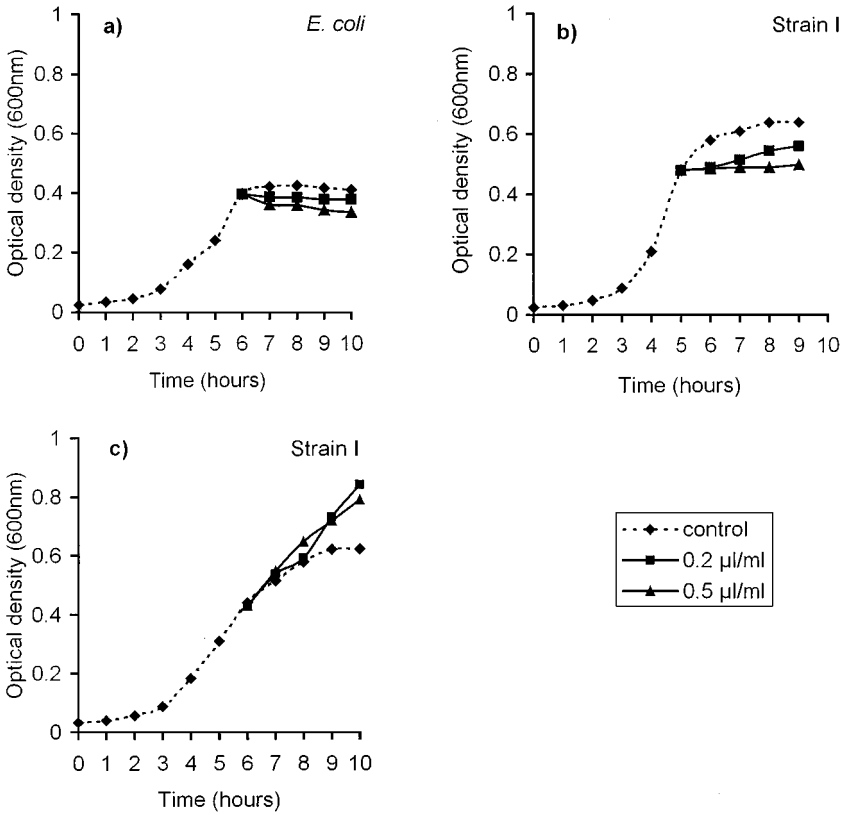


FIG. 5. Growth rate *E. coli* (a), strain II (b), and of strain I (c) before and after addition of fenchone (two quantities tested); the compound was added when populations reached the stationary phase of their growth. Experiments were repeated twice. Each value in the graphs is the average of two; individual values differed by less than 3% from the mean.

Activation of soil respiration in the presence of *L. stoechas* essential oil or fenchone was accompanied by an increase in the soil bacterial population, of approximately three orders of magnitude. This suggests that increased soil respiration can be attributed to increased bacterial populations in the soil and their concomitant metabolic activity. This population was qualitatively different from control soil samples. One bacterial strain in particular (I, Table 2) had an overwhelming participation in treated soils. These results support our working hypothesis that essential oils shift the microbial population balance in soil favoring particular bacterial strains.

Strain I, dominant in treated soils, was most resistant in the disk diffusion assays. The overall rank of bacterial resistance to monoterpenoids was: strain I > strain II ≥ strain III ≥ *E. coli* > *B. subtilis*. Of the compounds tested, fenchone and cineol were far more inhibitory than α-pinene. Oxygenated and nonoxygenated monoterpenoids repeatedly have been reported to differ in activity. Hydrocarbons (such as α-pinene) have been found to be far less active than oxygenated compounds (Sivropoulou, 1996; Karamanoli et al., 2000).

E. coli and *B. subtilis* have often been target organisms in assays examining the antimicrobial activity of various monoterpenoids (Pellecuer et al., 1980; Janssen et al., 1984; Yun et al., 1993; Sivropoulou et al., 1995, 1996). *B. subtilis* has always proved to be most sensitive, as in this study. In contrast, different patterns of response by *E. coli* strains have been reported, even when testing the same compounds.

Pseudomonas strains have been found to be resistant to essential oils and their individual constituents, (Janssen et al., 1984; Kivanç and Akgül, 1986; Kubo et al., 1992; Sivropoulou et al., 1996), and also able to use isoprenoids as a carbon and energy source (Dhavalikar and Bhattacharya, 1966; Gibbon and Pirt, 1971; Gunsalus and Marshall, 1971). However, this does not always hold true, as sensitive strains have been reported (Kivanç and Akgül, 1986; Karamanoli et al., 2000).

Strain II, tentatively identified as *Pseudomonas putida*, which we isolated from the treated soil samples, was only weakly affected. In fact, long ago it was found that this bacterial strain could degrade camphor (Gunsalus and Marshall, 1971), an oxygenated isoprenoid that is also present in *L. stoechas* essential oil. The authors reported that prominent among the organisms capable of attacking hydrophobic residues were soil saprophytic bacteria of the genus *Pseudomonas*. Inducible hydrogenases were found in *P. putida*, the model system that they studied. Some of these enzymes were not formed until other primary carbon sources were exhausted. This may explain why some essential oils and individual constituents do not cause an immediate effect, but require a certain period of time before increasing soil respiration (Vokou et al., 1984; Vokou and Margaris, 1988). The genes encoding the camphor catabolic enzymes were mapped on transmissible plasmids (Gunsalus and Marshall, 1971). If widespread, this genetic arrangement might provide a mechanism by which diverse compounds found in nature (i.e., isoprenoids) could be catabolized. With the results of this work, we cannot argue that *P. putida* can also metabolize fenchone. However, we can classify it among the bacteria resistant to *L. stoechas* essential oil and its constituents.

The striking participation of strain I in the treated soils, its ability to grow on/in solid and liquid media in presence of isoprenoids, and the fact that addition of fenchone at the end of the exponential phase of its growth caused further increases in its population size all indicate that it is able to biodegrade these secondary

metabolites. From the latter experiment, we can confidently state that strain I is resistant to fenchone and can use it as both a carbon and energy source.

On the basis of these results, we contend that essential oils determine the successional stages during the decomposition process of the litter of aromatic plants. Bacteria (e.g., strain I), able to use essential oils as carbon sources, must be among the initial colonizers of the rich-in-essential-oil litter (Vokou, 1992) from these plants. Activated in autumn, after the first rainfalls that follow the dry Mediterranean summer, these bacteria grow using essential oils as their carbon and energy sources. Among the later colonizers must be bacterial strains inhibited by these toxic substrates; they do not establish in the litter until the initial colonizers begin consuming the essential oils. Given that strains II and III were only weakly affected and were present in treated soil samples, we suggest that they may also participate in rather early successional stages. Whether the similar soil respiration response, induced by essential oils in all soil types that we have studied to date, is due to the same or a different spectrum of microorganisms is not known. This will be one of the directions of our future research, to understand the interactions between aromatic plants and soil microorganisms.

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REPTILIAN CHEMISTRY: VOLATILE COMPOUNDS FROM PARACLOACAL GLANDS OF THE AMERICAN CROCODILE (*Crocodylus acutus*)

SILVINA GARCÍA-RUBIO,[1] ATHULA B. ATTYGALLE,[1] PAUL J. WELDON,[2] and JERROLD MEINWALD*,[1]

[1]*Department of Chemistry and Chemical Biology*
Cornell University
Ithaca, New York 14853-1301

[2]*Conservation and Research Center*
Smithsonian Institution
1500 Remount Road
Front Royal, Virginia 22630

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Abstract—The secretion of the paracloacal glands of the American crocodile (*Crocodylus acutus*) contains over 80 lipophilic compounds, including saturated and unsaturated long-chain alcohols along with their formic, acetic, and butyric acid esters, and several isoprenoids. Most of these compounds were identified on the basis of mass spectra, obtained by GC-MS. In addition, identification of the major components was supported by infrared spectra obtained by GC-FTIR. Major differences are indicated in the composition of the paracloacal gland secretion of *C. acutus* and that of another crocodylid, the African dwarf crocodile (*Osteolaemus tetraspis*).

Key Words—American crocodile, *Crocodylus acutus*, paracloacal glands, fatty esters, formates, mass spectrometry, GC-FTIR.

INTRODUCTION

While insect chemical ecology has made great strides in the last five decades, much less is known about many comparable aspects of vertebrate chemical ecology (Eisner and Meinwald, 1995). The crocodilians, a small but fascinating group of ancient vertebrates, have aroused the curiosity of chemical ecologists in the last decade, with primary attention being paid to the chemical composition of their skin

* To whom correspondence should be addressed. E-mail: circe@cornell.edu

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gland secretions (Weldon and Wheeler, 2000). The study of crocodilian ecology and behavior, however, has lagged behind that of most other tetrapods. This circumstance can be attributed in part to the difficulties inherent in studying responses to chemicals by these large and, for adults at least, dangerous reptiles in field or seminatural settings. We have studied the chemistry of the secretion produced in the paracloacal glands of the American crocodile (*Crocodylus acutus*, Crocodylidae) in the hope that knowledge of this chemistry will ultimately contribute to an understanding of crocodilian chemical ecology. These glands in crocodilians are thought to produce pheromones involved in the nesting and/or mating activities (Weldon and Ferguson, 1993).

Previous analyses of lipids from the paracloacal glands of crocodilians have occasionally led to the characterization of unusual compounds, thus reflecting some novel biosynthetic capabilities of these reptiles (Weldon and Wheeler, 2000). For example, a unique 19-carbon aromatic ketone, named dianeackerone, and a family of related steroidal esters were found to be major glandular products of the African dwarf crocodile (*Osteolaemus tetraspis*, Crocodylidae) (Whyte et al., 1999; Yang et al., 1999, 2000). Two stereoisomers of dianeackerone occur in varying ratios in adults of this species, but this compound was not detected in immatures.

We report here the results of an analysis by gas chromatography–mass spectrometry (GC-MS) of the paracloacal gland secretion of another crocodylid, *C. acutus*. This species occurs in freshwater and brackish coastal habitats in Mexico, Central America, northern South America, Cuba, Jamaica, Hispaniola, and in the tip of southern Florida. Our study revealed that the glandular secretion of this species is composed largely of formate, acetate, and butyrate esters of C_{12}–C_{20} straight-chain alcohols, along with the alcohols themselves. Interestingly, formate esters have not previously been reported in reptiles. This is the first detailed report of the skin gland lipids of *Crocodylus*, the most speciose genus of crocodilians.

METHODS AND MATERIALS

Paracloacal gland secretions of *C. acutus* were obtained from one adult male [total length (TL) = 3.0 m], one adult female (TL = 2.5 m), six juvenile males (TL = 1–1.5 m), five juvenile females (TL = 0.7–1.1 m), and five unsexed hatchlings (TL = 0.5–0.6 m) maintained at Cypress Gardens (Plant City, Florida), Gatorama (Palmdale, Florida), and the St. Augustine Alligator Farm (St. Augustine, Florida). The adults we sampled, which had been housed together for 2.8 years, are within the size range of reproductively active, free-ranging *C. acutus* (Kushlan and Mazzotti, 1989); they had copulated, although neither egg-laying nor nesting behavior had been observed. Most secretion donors hatched from eggs laid by captive females from Florida; one juvenile female was reared from an egg laid by a female from Jamaica. Animals at Cypress Gardens were fed rats and chicken necks, those at Gatorama were fed a commercial feline food

(Nebraska Brand, Central Nebraska Packing Company, North Platte, Nebraska) containing primarily horse meat, and those at the St. Augustine Alligator Farm were fed nutria (*Myocastor coypu*).

The secretions were collected by manually pressing the region around the cloacal opening, causing the walls of the cloaca to flare outward, exposing the openings of the gland ducts. Additional pressure applied to the glandular region resulted in the discharge of yellow secretions from the glands. These exudates were collected in capillary tubes that were broken off into glass vials. A few milliliters of CH_2Cl_2 were added to the vials, which were stored on dry ice.

Determination of Position of Double Bonds. Dimethyl disulfide (DMDS) derivatization was carried out according to a reported procedure (Attygalle and Morgan, 1986).

Reference Compounds. Fatty acids and alcohols, cholesterol, cholesta-3,5-diene, citronellol, pentadecane, and squalene were purchased from Sigma-Aldrich. Cholesteryl and alkyl formates were synthesized by the uncatalyzed reaction of each alcohol with an excess of formic acid (Burger et al., 1996). Acetates were synthesized by treating each alcohol with an excess of acetic anhydride and pyridine (Zhdanov and Zhenodarova, 1975). The reaction of the alcohols with butyryl, caproyl, and capryl chloride in CH_2Cl_2 afforded the corresponding butyrates, caproates, and caprylates, respectively (Ihara et al., 1990; Madden and Prestwich, 1994). 2,3-Dihydrofarnesol was prepared following a literature procedure (Kaplay et al., 1980).

Analytical Procedures. Gas chromatography was performed on a Hewlett-Packard (HP) 5890 instrument equipped with a splitless injector, and either a flame-ionization detector, a HP 5965A IR detector, or a HP 5970 mass selective detector. The samples were injected in the splitless mode. Analyses were performed using a 25-m $\times$ 0.22-mm fused silica-column coated with SE-54. The oven was programmed from 40°C (2 min) at 7°C/min to 280°C (30 min). To investigate the possible presence of nonvolatile compounds, we undertook a direct analysis of unfractionated secretion samples by NMR spectroscopy, as well as by electrospray ionization–mass spectrometry. No additional compounds were found with these methodologies.

RESULTS AND DISCUSSION

An exploratory survey of the paracloacal gland secretions of 21 crocodilians by thin-layer chromatography showed the presence of bands consistent with aliphatic esters in most taxa, including *Crocodylus* spp. (Weldon and Tanner, 1991). Analyses by GC-MS have revealed the structures of these esters in alligators (*Alligator* spp.) and caimans (*Caiman* spp. and *Paleosuchus* spp.) (Shafagati et al., 1989; Dunn et al., 1993; Ibrahim et al., 1998). The present study confirms the presence of many aliphatic acetates in *C. acutus*, and demonstrates the occurrence

of formates (characterized by *m/z* 47 ions) and butyrates (characterized by *m/z* 89 ions) as well. Moreover, the ester derived from 1-hexadecyl alcohol is the main component of the caproic acid esters; 1-hexadecyl caproate (*m/z* 117 and 224 ions) is also abundant in the secretions of the Chinese alligator (*A. sinensis)* (Dunn et al., 1993). Our analytical results are presented in Tables 1 and 2 and Figures 1 and 2.

We observed significant quantities of formate esters derived from C_{13}–C_{18} alcohols; the major formates were those of C_{16}, C_{17}, and C_{18} monounsaturated alcohols. The presence of formates is interesting since they have not been reported previously from crocodilians or from any other reptiles. While formates are known from ants (Lopez et al., 1993), beetles (Attygalle et al., 1992), and caterpillars (Attygalle et al., 1993), to the best of our knowledge they have not been reported among vertebrates except in the preorbital gland secretions of three species of African antelopes (Burger et al., 1981, 1996, 1999; Mo et al., 1995). The alcoholic moieties of these esters range, among the three antelope species, from 9 to 28 carbons. As Burger et al. (1981) noted, the mass spectra of formates are very similar to those of the corresponding aliphatic alcohols, but are distinguished from them by the presence of a low-intensity peak at *m/z* 47, corresponding to an $HCO_2H_2^+$ (protonated formic acid) fragment.

We observed free alcohols in the *C. acutus* ranging from 10 to 18 carbons, including the terpene citronellol, which was found only in the secretion of the hatchlings. A similar array of alcohols has been reported in some alligatorids, e.g.,

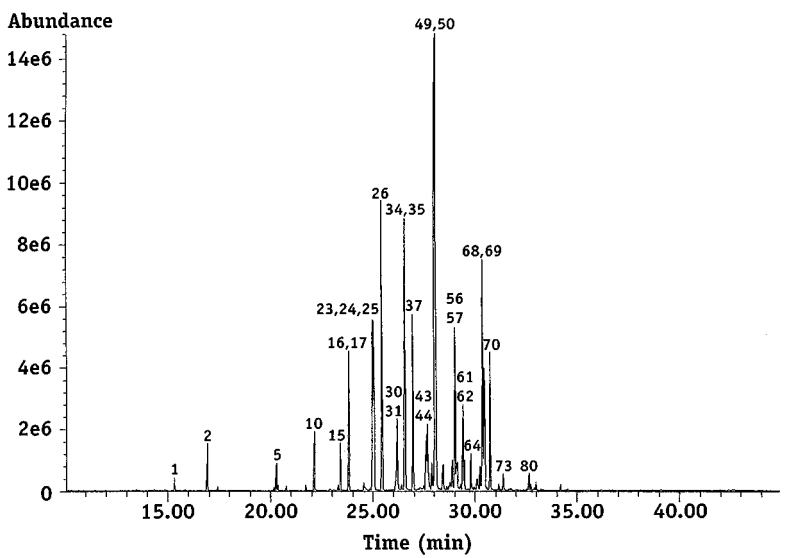


FIG. 1. A reconstructed gas chromatogram obtained from the paracloacal gland secretion of hatchling *Crocodylus acutus*. Peak numbers refer to components listed in Table 1.

TABLE 1. COMPONENTS FOUND IN PARACLOACAL GLAND SECRETION OF *Crocodylus acutus*[a]

No.	Compound	Molecular formula	NMM	Characteristic ions observed (*m/z*)	R_t (min)
1	Citronellyl formate	$C_{10}H_{19}OCHO$	184	138, 123, 95, 81, 69, 41	15.33
2	Citronellyl acetate	$C_{10}H_{19}O_2C_2H_3$	198	138, 123, 95, 81, 69, 43, 41	16.92
3	Pentadecane	$C_{15}H_{32}$	212	212, 71, 57, 43	20.11
4	Unidentified terpenoid			181, 135, 109, 69	20.17
5	Citronellyl butyrate	$C_{10}H_{19}O_2C_4H_7$	226	138, 123, 95, 81, 69, 43, 41	20.28
6	Dodecyl formate	$C_{12}H_{25}OCHO$	228	168	20.30
7	Tridecyl alcohol	$C_{13}H_{27}OH$	200	182	21.15
8	Dodecyl acetate	$C_{12}H_{25}OCHO$	228	168	21.71
9	Tridecyl formate	$C_{13}H_{27}OCHO$	228	182	22.12
10	Unidentified terpenoid			195, 123, 69, 41	22.19
11	Tetradecyl alcohol	$C_{14}H_{29}OH$	214	196	22.87
12	Unidentified terpenoid			209, 149, 123, 81, 69, 43, 41	23.30
13	Tetradecenyl formate[1]	$C_{14}H_{27}OCHO$	240	194	23.41
14	Tridecyl acetate	$C_{13}H_{27}O_2C_2H_3$	242	182	23.41
15	Unidentified terpenoid			209, 123, 69, 41	23.81
16	Tetradecyl formate	$C_{14}H_{29}OCHO$	242	196	23.81
17	Myristic acid	$C_{13}H_{27}CO_2H$	228	185	24.21
18	Unidentified terpenoid			195, 143, 171	24.21
19	Pentadecyl alcohol	$C_{15}H_{31}OH$	228	210, 182	24.52
20	Tetradecenyl acetate[1]	$C_{14}H_{27}O_2C_2H_3$	256	194	24.62
21	Unidentified		252	252, 135, 121, 79, 67,41	24.88
22	Dihydrofarnesyl acetate	$C_{15}H_{27}O_2C_2H_3$	266	223, 163109, 95, 81, 69, 43, 41	24.93
23	Tetradecyl acetate	$C_{14}H_{29}O_2C_2H_3$	256	196	25.01
24	Pentadecenyl formate[b]	$C_{15}H_{29}OCHO$	254	208	25.01
25	Pentadecyl formate	$C_{15}H_{31}OCHO$	256	210	25.42
26	Hexadecenyl alcohol[b]	$C_{16}H_{31}OH$	240	222	25.71
27	Pentadecanoic acid	$C_{14}H_{29}CO_2H$	242	199	25.71
28	Pentadecadienyl acetate[b]	$C_{15}H_{27}O_2C_2H_3$	266	206	25.97
29	Hexadecyl alcohol	$C_{16}H_{33}OH$	242	224	26.11
30	Pentadecenyl acetate[b]	$C_{15}H_{29}O_2C_2H_3$	268	208	26.18
31	Tridecyl butyrate	$C_{13}H_{27}O_2C_4H_7$	270	182	26.23
32	Unidentified		266	266, 135, 121, 81, 67, 41	26.39
33	7-Hexadecenyl formate[b]	$C_{16}H_{31}OCHO$	268	222	26.55
34	Pentadecyl acetate	$C_{15}H_{31}O_2C_2H_3$	270	210	26.55
35	Methyl palmitate	$C_{15}H_{31}CO_2CH_3$	270	227, 270	26.80
36	Hexadecyl formate	$C_{16}H_{33}OCHO$	270	224	26.94
37	Heptadecadienyl alcohol[b]	$C_{17}H_{31}OH$	252	234	27.15
38	Palmitic acid	$C_{15}H_{31}CO_2H$	274	256	27.22
39	Heptadecenyl alcohol[b]	$C_{17}H_{33}OH$	254	236	27.38
40	Heptadecyl alcohol	$C_{17}H_{35}OH$	256	238	27.48
41	Hexadecadienyl acetate[b]	$C_{16}H_{29}O_2C_2H_3$	280	220	27.48
42	Unidentified terpenoid			251, 191, 163, 81, 69	27.61
43	Hexadecenyl acetate[c]	$C_{16}H_{31}O_2C_2H_3$	282	222	27.61
44	Tetradecenyl butyrate[b]	$C_{14}H_{27}O_2C_4H_7$	282	194	27.64

TABLE 1. CONTINUED

No.	Compound	Molecular formula	NMM	Characteristic ions observed (*m/z*)	R_t (min)
45	Tetradecyl butyrate	$C_{14}H_{29}O_2C_4H_7$	284	196	27.69
46	Heptadecadienyl formate[b]	$C_{17}H_{31}OCHO$	282	234	27.78
47	Unidentified		280	280, 135, 121, 81, 67, 41	27.87
48	Hexadecyl acetate	$C_{16}H_{33}O_2C_2H_3$	284	224	28.02
49	Heptadecenyl formate[b]	$C_{17}H_{33}OCHO$	282	236	28.02
50	Heptadecyl formate	$C_{17}H_{35}OCHO$	284	238	28.42
51	Octadecenyl alcohol[d]	$C_{18}H_{35}OH$	268	250	28.70
					28.77
52	Pentadecenyl butyrate[b]	$C_{15}H_{29}O_2C_4H_7$	296	208	28.81
53	Unidentified terpenoid			169, 109, 69, 41	28.87
54	Heptadecadienyl acetate[b]	$C_{17}H_{31}O_2C_2H_3$	294	234	28.87
55	Heptadecenyl acetate[e]	$C_{17}H_{33}O_2C_2H_3$	296	236	29.02
					29.10
56	Octadecyl alcohol	$C_{18}H_{37}OH$	270	252	29.10
57	Pentadecyl butyrate	$C_{15}H_{31}O_2C_4H_7$	298	210	29.10
58	Unidentified		294	294, 135, 121, 81, 67, 41	29.28
59	Heptadecyl acetate	$C_{17}H_{35}O_2C_2H_3$	298	238	29.40
60	Octadecenyl formate[f]	$C_{18}H_{35}OCHO$	296	250	29.40
					29.48
61	Oleic acid	$C_{17}H_{33}CO_2H$	282	264	29.69
62	Octadecyl formate	$C_{18}H_{37}OCHO$	298	252	29.79
63	Stearic acid	$C_{17}H_{35}CO_2H$	284	266	30.07
64	Hexadecenyl butyrate[b]	$C_{16}H_{31}O_2C_4H_7$	310	222	30.07
65	Octadecadienyl acetate[b]	$C_{18}H_{33}O_2C_2H_3$	308	248	30.09
					30.24
66	Octadecenyl acetate[g]	$C_{18}H_{35}O_2C_2H_3$	310	250	30.36
					30.42
67	Hexadecyl butyrate	$C_{16}H_{33}O_2C_4H_7$	312	224	30.42
68	Octadecyl acetate	$C_{18}H_{37}O_2C_2H_3$	312	252	30.72
69	Unidentified		308	308, 135, 121, 81, 67, 41	31.17
70	Heptadecadienyl butyrate[1]	$C_{17}H_{33}O_2C_4H_7$	322	234	31.35
71	Heptadecenyl butyrate[b]	$C_{17}H_{33}O_2C_4H_7$	324	236	31.37
72	Pentadecyl caproate	$C_{15}H_{31}O_2C_6H_{11}$	328	177, 210	31.65
73	Nonadecenyl acetate[b]	$C_{19}H_{37}O_2C_2H_3$	324	264	31.66
74	Heptadecyl butyrate	$C_{17}H_{35}O_2C_4H_7$	326	238	31.73
75	Eicosenyl formate[b]	$C_{20}H_{39}OCHO$	324	278	32.00
76	Nonadecyl acetate	$C_{19}H_{39}O_2C_2H_3$	326	266	32.00
77	Octadecadienyl butyrate[b]	$C_{18}H_{33}O_2C_4H_7$	336	248	32.37
78	Octadecenyl butyrate[h]	$C_{18}H_{35}O_2C_4H_7$	338	250	32.64
					32.72
79	Eicosenyl acetate[b]	$C_{20}H_{39}O_2C_2H_3$	338	278	32.88
80	Hexadecyl caproate	$C_{16}H_{33}O_2C_6H_{11}$	340	177, 224	32.88
81	Octadecyl butyrate	$C_{18}H_{37}O_2C_4H_7$	340	252	32.98
82	Eicosyl acetate	$C_{20}H_{41}O_2C_2H_3$	340	280	33.23
83	Unidentified terpenoid			376, 333, 109, 69	34.18

TABLE 1. CONTINUED

No.	Compound	Molecular formula	NMM	Characteristic ions observed (*m/z*)	R_t (min)
84	Hexadecyl caprylate	$C_{16}H_{33}O_2C_8H_{15}$	368	368, 224, 145	35.13
85	Octyl oleate	$C_8H_{17}O_2C_{18}H_{33}$	394	264	37.52
86	Squalene	$C_{30}H_{50}$	410	41, 69, 81	38.09
87	Cholesta-3,5-diene	$C_{27}H_{44}$	368	368, 353, 147	39.06
88	Dodecyl oleate	$C_{12}H_{25}O_2C_{18}H_{33}$	450	264	40.70
89	Cholesterol	$C_{27}H_{46}O$	386	386	42.54
90	Cholesteryl formate	$C_{27}H_{45}OCHO$	414	368	43.95

[a] Compounds are numbered in order of increasing GC retention time. NMM = nominal molecular mass. Trace components are not listed.
[b] Double bond position undetermined.
[c] Mixture of (*Z*)-hexadec-7-en-1-yl acetate (major) and (*Z*)-hexadec-9-en-1-yl acetate (minor).
[d] Mixture of (*Z*)-octadec-9-en-1-ol (major) and (*Z*)-octadec-11-en-1-ol (minor).
[e] Mixture of (*Z*)-heptadec-8-en-1-yl acetate (major) and (*Z*)-heptadec-9-en-1-yl acetate (minor).
[f] Mixture of (*Z*)-octadec-9-en-1-yl formate (major) and (*Z*)-octadec-11-en-1-yl formate (minor).
[g] Mixture of (*Z*)-octadec-9-en-1-yl acetate (major) and (*Z*)-octadec-11-en-1-yl acetate (minor).
[h] Mixture of (*Z*)-octadec-9-en-1-yl butyrate (major) and (*Z*)-octadec-11-en-1-yl butyrate (minor).

the Chinese alligator (*A. sinensis*) (Dunn et al., 1993) and caimans (*Paleosuchus* spp.) (Shafagati et al., 1989), although citronellol has been described only from several caimans. 2,3-Dihydrofarnesyl acetate, a known paracloacal gland component of the brown caiman (*Caiman crocodilus fuscus*) (Wheeler et al., 1999), accounted for up to 4% of the secretion of immature crocodiles, but was not detected in the adult samples.

Interestingly, we did not find free formic, acetic, or butyric acids in any of the secretions, based on the derivatization of the total sample with pentafluorobenzyl bromide/triethylamine (Attygalle, 1998). Five C_{14}–C_{18} fatty acids, which are routinely encountered from vertebrate integumentary organs, were observed in immature *C. acutus*, and a significant amount of oleic acid (~5%) was found in the adult male; we failed to detect free fatty acids in hatchling *C. acutus*. Thirteen fatty acids ranging from C_{14} to C_{20} were reported from the skin glands of *Crocodylus* spp. by Navajas Polo et al. (1988a,b). It should be noted that these investigators combined, for their studies, the paracloacal and gular (throat) glands of both *C. acutus* and the Cuban crocodile (*C. rhombifer*) thus our results are not directly comparable with theirs. We were also unable to confirm the presence of an intriguing lactone related to 10-hydroxystearic acid described in an earlier report (Navajas Polo, 1982) from the macerated and steam-distilled skin glands of the aforementioned mixed species.

Squalene was the major hydrocarbon we detected in *C. acutus*. This compound was especially abundant in the adult female; it was either present in reduced

TABLE 2. RELATIVE AMOUNTS OF COMPOUNDS IDENTIFIED FROM PARACLOACAL GLANDS OF HATCHLING, IMMATURE, AND ADULT *Crocodylus acutus*[a]

Compound	Hatchlings (5)	Immatures		Adults	
		Female (5)	Male (6)	Female (1)	Male (1)
Alkyl and alkenyl esters					
Dodecyl formate	<0.2	<0.2	<0.2	ND	ND
Tridecyl formate	1.30	<0.2	0.32	ND	ND
Tetradecenyl formate	<0.2	<0.2	<0.2	ND	<0.2
Tetradecyl formate	2.07	0.24	0.73	ND	0.63
Pentadecenyl formate	1.10	<0.2	0.31	ND	0.30
Pentadecyl formate	8.16	1.09	2.91	ND	2.50
7-Hexadecenyl formate	1.30	0.57	1.83	0.51	2.07
Hexadecyl formate	4.44	1.53	4.92	0.96	6.89
Heptadecadienyl formate	ND	ND	<0.2	ND	ND
Heptadecenyl formate	4.12	2.53	0.77	<0.2	9.26
Heptadecyl formate	0.59	<0.2	0.91	ND	0.89
Octadecenyl formate	2.31	3.73	11.28	1.67	20.06
Octadecyl formate	0.99	0.61	2.62	1.39	4.80
Eicosenyl formate	ND	<0.2	<0.2	ND	<0.2
Dodecyl acetate	<0.2	<0.2	<0.2	ND	ND
Tridecyl acetate	1.02	0.45	0.31	ND	ND
Tetradecenyl acetate	<0.2	<0.2	<0.2	ND	ND
Tetradecyl acetate	3.82	1.65	1.14	<0.2	<0.2
Pentadecadienyl acetate	ND	<0.2	<0.2	ND	ND
Pentadecenyl acetate	1.90	0.64	0.71	ND	ND
Pentadecyl acetate	7.25	3.48	3.28	ND	0.86
Hexadecadienyl acetate	<0.2	<0.2	<0.2	ND	ND
Hexadecenyl acetate	2.06	1.21	1.72	0.97	0.61
Hexadecyl acetate	19.97	10.85	9.47	4.04	3.59
Heptadecadienyl acetate	1.02	2.02	1.79	ND	ND
Heptadecenyl acetate	5.36	6.13	8.90	2.51	4.31
Heptadecyl acetate	0.73	0.79	0.84	1.67	0.34
Octadecadienyl acetate	1.15	4.10	2.19	ND	6.09
Octadecenyl acetate	9.10	14.40	13.84	7.03	9.90
Octadecyl acetate	3.30	3.73	4.29	4.58	2.28
Nonadecenyl acetate	ND	<0.2	<0.2	ND	ND
Nonadecyl acetate	ND	<0.2	<0.2	ND	ND
Eicosenyl acetate	<0.2	<0.2	<0.2	ND	ND
Eicosyl acetate	<0.2	<0.2	<0.2	ND	ND
Tridecyl butyrate	<0.2	<0.2	0.23	ND	ND
Tetradecenyl butyrate	ND	ND	<0.2	ND	ND
Tetradecyl butyrate	0.32	0.36	0.54	<0.2	<0.2
Pentadecenyl butyrate	<0.2	ND	<0.2	ND	ND
Pentadecyl butyrate	0.60	0.93	0.74	ND	0.45
Hexadecenyl butyrate	<0.2	<0.2	0.28	ND	0.76
Hexadecyl butyrate	1.98	1.11	2.09	2.25	0.76
Heptadecadienyl butyrate	<0.2	<0.2	<0.2	ND	ND

TABLE 2. CONTINUED

Compound	Hatchlings (5)	Immatures		Adults	
		Female (5)	Male (6)	Female (1)	Male (1)
Heptadecenyl butyrate	0.422	0.33	0.69	ND	0.88
Heptadecyl butyrate	<0.2	<0.2	<0.2	ND	0.42
Octadecadienyl butyrate	ND	<0.2	<0.2	ND	ND
Octadecenyl butyrate	0.61	0.64	0.98	<0.2	3.30
Octadecyl butyrate	0.20	0.40	<0.2	<0.2	0.94
Pentadecyl caproate	<0.2	<0.2	ND	ND	ND
Hexadecyl caproate	<0.2	<0.2	<0.2	ND	ND
Hexadecyl caprylate	ND	<0.2	ND	ND	ND
Methyl palmitate	ND	<0.2	<0.2	ND	ND
Octyl oleate	ND	ND	<0.2	ND	ND
Dodecyl oleate	ND	<0.2	<0.2	ND	ND
Terpenoid Esters					
Citronellyl formate	0.26	ND	ND	ND	ND
Citronellyl acetate	1.05	ND	ND	ND	ND
Citronellyl butyrate	0.66	ND	<0.2	ND	ND
Dihydrofarnesyl acetate	4.75	0.63	0.41	<0.2	ND
Alcohols					
Tridecyl alcohol	ND	<0.2	ND	ND	0.22
Tetradecyl alcohol	ND	0.23	<0.2	ND	ND
Pentadecyl alcohol	0.23	1.16	0.68	ND	ND
Hexadecenyl alcohol	ND	<0.2	<0.2	ND	ND
Hexadecyl alcohol	0.58	2.30	1.24	ND	ND
Heptadecadienyl alcohol	ND	<0.2	0.26	0.26	ND
Heptadecenyl alcohol	<0.2	0.26	1.23	ND	ND
Heptadecyl alcohol	ND	<0.2	<0.2	ND	ND
Octadecenyl alcohol	0.46	1.00	2.30	ND	ND
Octadecyl alcohol	ND	0.37	<0.2	ND	ND
Acids					
Myristic acid	ND	<0.2	<0.2	ND	ND
Pentadecanoic acid	ND	<0.2	<0.2	ND	ND
Palmitic acid	ND	<0.2	0.35	ND	ND
Oleic acid	ND	0.22	0.88	ND	4.89
Stearic acid	ND	<0.2	<0.2	ND	ND
Hydrocarbons					
Pentadecane	ND	<0.2	ND	ND	ND
Squalene	ND	3.44	0.74	52.00	0.33
Steroids					
Cholesta-3,5-diene	ND	<0.2	<0.2	ND	ND
Cholesterol	ND	0.27	<0.2	ND	<0.2
Cholesteryl formate	ND	<0.2	<0.2	ND	ND
Unidentified compounds					
Terpenoid No. 4	<0.2	0.45	<0.2	<0.2	ND
Terpenoid No. 10	<0.2	<0.2	<0.2	ND	ND
Terpenoid No. 12	<0.2	<0.2	<0.2	ND	ND

TABLE 2. CONTINUED

Compound	Hatchlings (5)	Immatures		Adults	
		Female (5)	Male (6)	Female (1)	Male (1)
Terpenoid No. 15	1.37	0.27	0.52	ND	ND
Terpenoid No. 18	ND	ND	<0.2	ND	ND
Unidentified No. 21	ND	<0.2	<0.2	ND	ND
Unidentified No. 32	0.25	<0.2	0.27	ND	ND
Unidentified No. 42	1.50	<0.2	<0.2	ND	ND
Unidentified No. 47	0.78	0.71	1.61	ND	2.46
Unidentified No. 53	<0.2	<0.2	ND	ND	ND
Unidentified No. 58	<0.2	0.54	0.77	ND	3.00
Unidentified No. 69	<0.2	<0.2	<0.2	ND	ND
Terpenoid No. 83	<0.2	ND	<0.2	ND	ND

[a] Numbers in parentheses indicate the number of individuals sampled. ND = non detected.

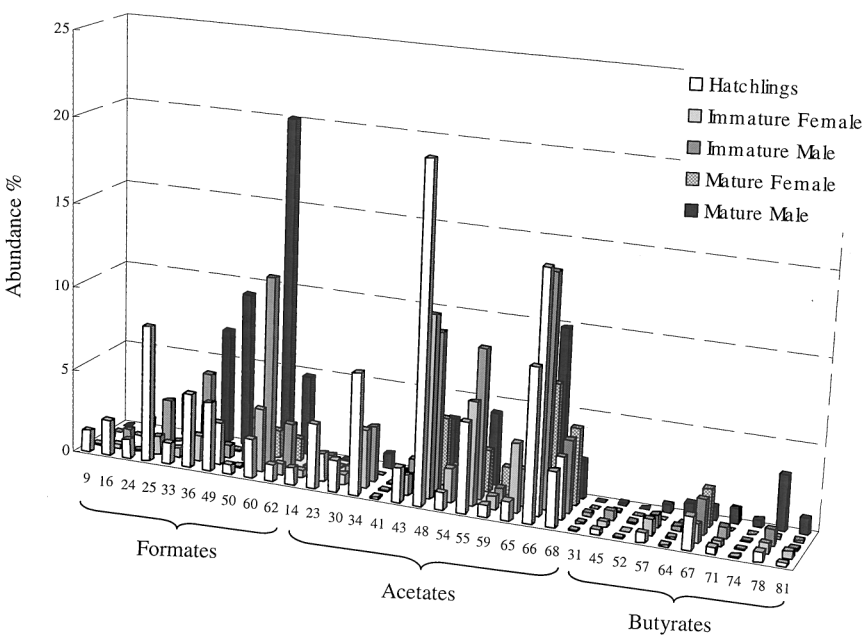


FIG. 2. Distribution of the alkyl and alkenyl esters (formates, 9–62; acetates, 14–68; and butyrates, 31–81) found in average abundance >0.2% in the paracloacal gland secretion of *Crocodylus acutus*, expressed as a percentage of the total secretion. Percentages are listed in Table 2; numbers refer to components listed in Table 1. Crocodile hatchlings were not sexed.

quantities or was entirely absent in the male or immature females. The abundance of squalene in our adult female is consistent with the appearance of an intense band corresponding to hydrocarbons in the thin-layer chromatogram of a female *C. acutus* secretion sample examined in the earlier survey of paracloacal gland lipids by Weldon and Tanner (1991).

All our compound identifications are based on the mass spectra obtained by GC-MS and comparison with synthetic standards. In addition, the identification of the major components was supported by infrared spectra obtained by gas chromatography–Fourier transform IR spectroscopy. Gas-phase FTIR spectra proved particularly helpful in recognizing formate, acetate, and butyrate peaks. Thus, the carbonyl group stretching vibrations appeared characteristically at 1740–1748 cm^{-1} in the gas-phase FTIR spectra of C_{13}–C_{18} formates, whereas that of acetates and butyrates appeared at 1761–1762 and 1753–1754 cm^{-1}, respectively. FTIR spectra were also useful for the assignment of the *Z* configuration to the double bonds in the unsaturated esters and alcohols. Double-bond positions in 9- and 11-octadecenyl acetate, 8- and 10-heptadecenyl acetate, 7- and 9-hexadecenyl acetate, 9-octadecenyl formate, 8-heptadecenyl formate, and 7-hexadecenyl formate were determined by the DMDS derivatization method (Buser et al., 1983; Francis and Veland, 1981).

We did not observe in *C. acutus* either dianeackerone or the related steroidal esters characteristic of the confamilial *Osteolaemus tetraspis* (Whyte et al., 1999; Yang et al., 1999); conversely, neither the aliphatic esters, alcohols, nor squalene that we have observed in *C. acutus* have been found in *O. tetraspis*. These results, foreshadowed by earlier TLC studies (Weldon and Tanner, 1991; Weldon and Wheeler, 2000), demonstrate substantive chemical differences in the paracloacal gland products of *Crocodylus* and *Osteolaemus*. While further analyses of the glandular products of *Crocodylus* will be needed to assess the range of chemical variation within the Crocodylidae, we can anticipate that the deeper task of deciphering the biological significance of these chemically complex secretions will remain a challenge for some time to come.

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INFLUENCE OF MHC ON SIBLING DISCRIMINATION IN ARCTIC CHAR, *Salvelinus alpinus* (L.)

K. H. OLSÉN,[1,*] M. GRAHN,[2] and J. LOHM[3]

[1]*Department of Environmental Toxicology, EBC*
Uppsala University
Uppsala, Sweden

[2]*Department of Biosciences at Novum*
Karolinska Institutet
University College Södertörn, Sweden

[3]*Molecular Population Biology Laboratory*
Department of Animal Ecology
Lund University, Sweden

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Abstract—The preference of juvenile Arctic char [*Salvelinus alpinus* (L.)] for odors from siblings and nonsiblings with different major histocompability complex class II (MHC) genotypes was studied in two-choice fluviarium tests. In the first part of the study, test fish demonstrated no preference for water scented by a sibling with a MHC genotype different from its own versus water scented by a MHC identical nonsibling. When both donors were siblings with different MHC genotypes, however, the test fish chose the water scented by the fish with the same MHC type as the test fish. The results suggest that odors with information about kinship are dependent on MHC but also on other, unknown factors. In the second part of the study, we observed that fish isolated since fertilization did not show any behavioral discrimination towards siblings, based on MHC genotype. One reasonable explanation for this result is that Arctic char learn to discriminate between odors from individuals of different MHC types.

Key Words—Arctic char, fish, chemical signals, MHC, kin discrimination, siblings, odors.

*To whom correspondence should be addressed at current address: Department of Natural Sciences, University College, Södertörn, Box 4101, SE-141 04 Huddinge, Sweden. E-mail: hakan.olsen@sh.se

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INTRODUCTION

The ability to discriminate between kin and nonrelated individuals has been demonstrated in various taxonomic groups of animals, including fish (Fletcher and Michener, 1987; Hepper, 1991; Olsén, 1992; Brown and Brown, 1996). Hamilton (1964) suggested that an individual's fitness is not only composed of its own fitness, but also involves the fitness of close relatives, i.e., the inclusive fitness theory. The ability to recognize kin makes it possible for an animal to act toward relatives in a way that increases its fitness, i.e., kin selection is possible (Wilson, 1975). Direct kin recognition mechanisms are composed of inherited and/or acquired components. Waldman (1987, 1991) suggested that direct recognition is composed of series of events. An individual presents labels (often odors, phenotype) that are detected by a second individual and these labels are compared with a template that is innate or learned. Recognition occurs if the fit of the label to the template is over a certain threshold and, if the social context is appropriate, a behavioral discrimination occurs.

Juvenile salmonids are capable of discriminating between water scented by siblings and fish from other sibling groups of the same population (Quinn and Busack, 1985; Olsén, 1989; Brown et al., 1993). They are more attracted to water with sibling odor than to water with odors from nonkin, even if both odor donors are unfamiliar to the test fish. These results demonstrate that the discrimination is based on direct recognition by phenotypic matching. Recent studies have also shown that the fish need experience with conspecifics during some early learning period, to establish a template by which other individuals can be compared (Winberg and Olsén, 1992; Olsén and Winberg, 1996).

The function behind kin recognition and discrimination in salmonid fish is not fully understood. It has been observed, however, that the level of aggression in pure sibling groups is lower than in groups of mixed relationship (Brown and Brown, 1993a,b; Olsén et al., 1996; Olsén and Järvi, 1997). The size of feeding territories was also larger in the mixed kin groups (Brown and Brown, 1993a,b). Attraction to sibling specific odors may also indicate that the fish prefer to school with related individuals (Olsén, 1989). Recent studies have also shown that the growth rate in pure sibling groups was greater and more even, compared to groups of mixed relationship (Brown and Brown, 1993a, 1996). These studies give support to the idea that juvenile salmonids benefit from the ability to discriminate between kin and non-kin.

The substances used for kin discrimination in juvenile salmonids are not known. In an elegant electrophysiological study (EOG), Moore and collaborators (Moore et al., 1994) showed that juvenile Atlantic salmon release odors containing information about kinship in the urine. Further more, juvenile Arctic char are attracted to water scented by urine or intestinal content from individuals from the same population (Olsén, 1987). We recently demonstrated that the odors released

by juvenile Arctic char and used in kin discrimination are significantly influenced by the major histocompability complex (MHC class II) (Olsén et al., 1998). MHC molecules (class I and II) are used for recognition of antigens and self-reference is required for the recognition of nonself substances (Klein, 1986). Our results correspond well with studies with mice (*Mus musculus*) that show abilities to discriminate between the odors of conspecifics with disparate MHC genes. The odors are present in the urine (Yamazaki et al., 1976, 1979; Singh et al., 1987, 1988; Potts et al., 1991), and the recognition ability is probably based on a familiar imprinting process of kin odors (Penn and Potts, 1998a). Mice may use this information for kin recognition and/or to increase the heterozygosity of MHC genes that may result in increased resistance towards parasites (e.g., Beauchamp et al., 1988; Potts et al., 1991; Manning et al., 1992; Lewis, 1998; Penn and Potts, 1998b; Eggert, 1999). Our recent study with juvenile Arctic char also indicated that genes from another part of the genome are involved in the production of cues used for kin discrimination when the fish cannot rely on information from the MHC class II (Olsén et al., 1998). We observed that a test fish did not show any preference when choosing between water scented by a sibling with no MHC class II genes in common and a nonsibling that shared one allele with the test fish. However, the test fish preferred the water from a sibling if both donors had no MHC-alleles in common with the test fish. Similar results showing the significance of other factors than MHC have been obtained from studies with rodents (Lennington et al., 1988; Brown et al., 1989; Schellink et al., 1993; Eggert et al., 1996). The MHC may be a major genetic factor that influences the odors of individuality, but there are other factors such as diet, social status, and the animal's bacterial flora that, together with inherited components, are of importance for the odor identity, as has been shown for rodents (e.g., Brown et al., 1996; Schellink et al., 1997; Zavazava and Eggert, 1997).

In the present study, we continue our previous work concerning the influence of MHC genes on kin discrimination in Arctic char. What happens if the test fish have to choose between odors from an MHC-like nonsibling (both alleles in common) and an MHC-different sibling? Further, by using test fish kept in isolation since fertilization, we want to ascertain whether the behavior discrimination of MHC is an innate response or might be based on learning.

METHODS AND MATERIALS

Fish. We created two full-sibling families, by using two pairs of wild Arctic char caught in Lake Hornavan, Lapland, Sweden, just before the spawning period in the autumn of 1995. Before the artificial fertilization was done, ten adult individuals of each sex were MHC characterized to obtain suitable sibling groups. The mature adults were placed in tanks at a hatchery close to Lake Hornavan (Sälla Fiskodling, Porjus).

Within 1 hr of fertilization, egg lots from the two pairs, family 1 and family 2, were each divided by volume into two equal groups. In addition, single eggs from both families were individually placed in compartments, each with its own water inlet and outlet (Olsén and Winberg, 1996). The eggs that were isolated were transported to Uppsala on the same day as they were fertilized. We incubated the rest of the eggs of the four groups (1A, 1B, 2A, 2B) in separate hatching boxes and placed each box at the upstream end of separate troughs to avoid contact with odors from conspecifics. Yolk sac fry from these four groups were transported to Uppsala University during the spring of 1996 and placed in equivalent boxes in separate troughs supplied with aerated Uppsala tap water. Later, we transferred fish groups to fiberglass holding tanks. In all groups, the fish remained healthy throughout the experimental period and mortality was negligible. All fish were fed daily with the same kind of Astra-EWOS granulated food pellets (EST40) in the amount of 1–2% of the body fresh weight.

Fluviarium Tests. Experiments were conducted during the period November 19, 1997, to January 20, 1998, in a modified standard fluviarium (Höglund, 1961; Olsén and Höglund, 1985; Olsén, 1986, 1989), which is a two-choice maze with a laminar flow of aerated tap water (see Figure 1 in Olsén et al., 1998). Water from two identical 12-liter donor aquaria (A1 and A2) was supplied to opposite halves of the fluviarium and mixed into the main water current flowing downstream to the test area. We placed one test fish in the test area, measuring $46 \times 33 \times 11.5$ cm (length $\times$ width $\times$ depth), limited upstream and downstream by two fine-mesh plastic nets. During the experiments, the flow (range during the entire study: 380–410 ml/min; no significant difference in flow during a test between the two channels) from the two donor aquaria was switched automatically between the two halves of the test area at regular 90-min intervals. The donor aquaria were continously supplied with aerated tap water at a rate of 440–450 ml/min. The flow rate of the water in the fluviarium was 19 liters/min (9.5 liters/min on each half) and the water temperature in the fluviarium and in the donor aquaria was 7.7–9.9°C. There was no difference in water temperature between the two donor aquaria, and the temperature difference between the aquaria and the fluviarium water was always less than 1.0°C. We placed a video camera 33 cm above the test area and recorded the position of the test fish once every 2 sec by means of a video-computer-based image analysis system (Bjerselius et al., 1995; Olsén and Winberg, 1996). A flat rack of infrared 468 diodes, transmitting wavelengths longer than 750 nm (beyond the visual range of salmonide fish) (Bone et al., 1995), were placed under the test area and used as light sources for the camera. To prevent external disturbances, fluviarium tests were run after the ordinary working hours for most people at the department. A test started at 17:00 hr and ended at 05:00 hr the next morning. Between the experiments, we washed the test area, the donor aquaria, tubes and electromagnetic valves with water and 95% ethanol. In addition, we brushed the aquaria with a solution of ethanol and acetic acid (97:3).

In each fluviarium test, data from the last 60 min in each of the eight 90-min periods in a complete test were used to calculate a reaction value (*Rv*). *Rv* was calculated according to Olsén (1986) using the following equation:

$$Rv = [(N1 - N2)/(N1 + N2)] \times 100,$$

where $N1$ and $N2$ represent the number of observations in each side of the test area with water scented by a individual placed in A1 and A2, respectively (a *Rv* of 100 would imply a complete preference for water from A1 and a $Rv = -100$ a complete preference for A2). The test fish always had the same MHC genotype as the fish in A1, except in control tests where the odor donors in the two aquaria had the same MHC class II genotype. The arithmetic means of reactions values from identical tests are designated M*Rv*.

MHC Matching Experiment. To test if the recognition and the following behavioral discrimination is a matching process influenced by MHC-based olfactory cues available to the test fish, 36 juveniles from two sibling groups were selected, all with a body mass between 8.4 and 10.7 g. The two sibling groups chosen shared MHC alleles between the clutches. On October 10, 1997, we placed the fish in diagonally divided troughs measuring 45 × 25 × 25 cm (length × width × depth). As the fish were placed in the troughs, a small piece of the caudal fin was cut for analyzing their individual MHC genotype. There were two fish in each trough, separated by a transparent partition which allowed both visual and chemical, but not physical, contact. The 18 troughs were continuously supplied with aerated tap water. All fish were in good condition and were fed once per day. The lights turned on at 05:00 hr and off at 17:00 hr, respectively. Individuals that had been isolated since fertilization were not moved to these troughs, but were maintained in their original compartments. The size of the test fish isolated since fertilization varied between 7.8 and 13.4 g ($N = 6$).

We ran four types of tests in the fluviarium (experiments 1–4, see below). The test fish and the donors were always from different troughs, and they were all changed between tests. In experiments 1–3, we used each test fish with three different combinations of donors ($N = 5$). One test fish of each belonged to groups 1A (genotype *fh*), 2A (*fd*), and 2B (*fd*). Two test fish belonged to 1B (both *fh*). To reduce the risk of recognition by familiarity, neither of the donors were from the same original rearing group as the test fish (e.g., from 1A) and the test fish and one of the donors in the triplet were not from the same diagonally divided trough. The differences in weight between the two donors were the same in the different experiments (mean ± SD; experiment 1: 0.9 g ± 0.6; experiment 2: 0.9 g ± 0.6; experiment 3: 0.9 g ± 0.7; $N = 5$ for all). Fish isolated since fertilization were used only as test fish and only once (experiment 4). Three test fish of each belonged to sibling group 1 (one fish *fh* and two *dd*) and 2 (one fish *fd* and two *ah*). The same pair of donors was used only once. The weight of the donors varied between

9.1 and 12.9 g ($N = 12$) and the mean difference in weight between the pair of donors used was 1.2 g $\pm$ 0.7 ($N = 6$).

We placed fish in the donor aquaria and in the fluviarium 30 min before the test started, and the following experiments were performed: (1) In the control test all three individuals belonged to the same sibling group. The two donors, one in each aquarium, were MHC-II identical. (2) A test fish was exposed to water scented by a MHC-identical sibling (identical to the test fish) and a MHC-different sibling. The experiments is designated as "full sibs, same MHC vs. full sibs, different MHC." (3) Water was scented by a nonsibling and a sibling. The test fish had the same MHC genotype as the nonsibling, but was different from the sibling odor donor. Designated as "non-sibs, same MHC vs. full-sibs, different MHC." (4) The test fish had been isolated since fertilization. The donors were siblings to the test fish and one sibling had the same MHC genotype as the test fish, the other sibling had different MHC genotype.

Tissue Preparation and PCR. A small tissue sample, 20–30 mg, was taken from the caudal fin of each fish. We immediately put the cut fins (approx. 6 mm^2) in a freezer (-20°C). This procedure did not cause any great harm to the fish, and it was possible to use them later in behavioral experiments. First, we took a random sample from the different groups (1A, 1B, 2A, 2B) to examine what genotypes were present. All fish placed in physical isolation were analyzed before the fluviarium test. Genomic DNA was extracted and purified according to Laird et al. (1991).

The complete exon 2 of a MHC class II gene encoding the hypervariable antigen binding site (315 bp) was amplified by the polymerase chain reaction (PCR) using standard procedures, 100 ng of DNA template in 25 μl PCR reactions (Perkin Elmer). MHC class II was chosen because the greater number of DNA sequences from related salmonid species (Hordvik et al., 1993) for this loci facilitated the design of specific PCR primers and development of a genotyping method. The conditions of the PCR were as follows: denaturation for 2 min at 94°C, followed by 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 56°C, and primer extension for 30 sec at 72°C. The final extension was for 10 min at 72°C. When run on an agarose gel, the PCR products showed one distinct band corresponding to 315 bp and high DNA homology (96%) to salmon cDNA (M. Grahn and J. Lohm, unpublished data). The PCR was performed on a Perkin-Elmer-Cetus 9600 instrument. The two primers used to amplify the class II B exon were MG7 (5′-TAATCTGACAAAACAATGA-3′) and MG14 (5′-GCGGGCGGCGCGGGGCGCGGGCAGGGCGGCGGGGGCGGGCCACC TGTCTTGTCCAGTATG-3).

Denaturing Gradient Gel Electrophoresis. To detect allelic differences within and between individuals, we used denaturing gradient gel electrophoresis (DGGE), which is used to separate different DNA fragments of the same length based on sequence differences (Fischer and Lerman, 1983; Myers et al., 1987). The combination of PCR and DGGE has previously been used to study MHC class II B gene

variation in primates and provides an easy and reliable method for MHC typing in new and less well-studied species compared to serological typing or restriction fragment length polymorphism, RFLP (Knapp et al., 1997).

DGGE was performed by using 5%, 19:1 acrylamide–bisacrylamide, polyacrylamide parallel gels containing 1× TAE buffer and a gradient of urea and formamide. Denaturant concentrations in the gels ranged from 30% to 60% (Myers et al., 1987). Electrophoresis was performed in 1× TAE buffer heated to 60°C in electrophoresis cells and a buffer tank (C.B.C. Scientific Company, Inc.). A volume of 10–15 μl PCR products per lane was electrophoresed between 5 hr and 6 hr at 180 V. The gels were stained with ethidium bromide (0.2 μg/ml 1× TAE) for 15 min and the DNA was visualized with a UV transilluminator (UVP, inc Transilluminator, Nagle Company).

Sequencing. To verify that the DNA sequences of identical DGGE bands were the same among unrelated individuals, the DGGE fragments were sequenced by direct sequencing. Desired DGGE fragments were cut out from the gel, mixed with 160 μl dd H_2O, extracted by keeping the samples for 1 hr at −80°C, 1 hr at 25°C, 1 hr at −80°C, 2 hr at 4°C and used as template in a PCR with the primers and annealing temperatures as above. The PCR products were ethanol precipitated, dissolved in 15 μl dd H_2O and used as sequencing template. Fragments were sequenced in both directions using the primers MG 7 and ÅL1002 (Langefors et al., 2000). Sequences were then analyzed in a ABI PRISM 310 (Perkin Elmer).

Statistics. Experiments 1–3 were compared with repeated-measures ANOVA followed by Tukey-Kramer multiple comparison test. Student's t test ($t = \mathrm{M}Rv/\mathrm{SD}/\sqrt{N}$) was used to compare the M*Rv* for each experiment with the theoretically indifferent reaction when $\mathrm{M}Rv = 0$. M*Rv* = 0 when the number of observations in each water quality is the same. All calculations were performed with GraphPadTM Instat.

RESULTS

To test if fish preferences were affected by MHC genotype, we used triplets of genotyped fish. The genotypes of the char in family 1 were *df*, *dd*, *fh*, and *dh* and in family 2 *df*, *fh*, *ad*, and *ah*. The alleles designated with the same letters in both families (i.e., d, f, and h) showed the same DNA sequences in both families (data not shown). The repeated measures ANOVA revealed significant differences between the reaction in the control test, the test with MHC-different siblings as donors (experiment 2, "non-sibs, same MHC vs. full-sibs, different MHC"), and the test with donors from two different sibling groups with the non-sibling MHC-like the test fish (experiment 3, "non-sibs, same MHC vs. full-sibs, different MHC")($F_{2,8} = 5.44$, $P < 0.05$; Figure 1). The Tukey-Kramer multiple comparisons test showed that there was a significant difference between the reactions in

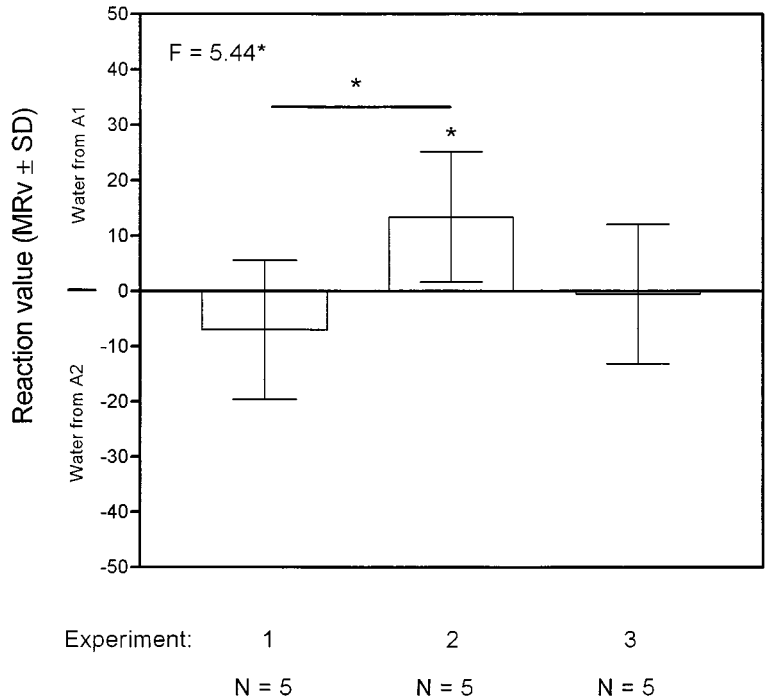


FIG. 1. Reaction value (M*Rv* ± SD) of juvenile Arctic char exposed to water scented by an unfamiliar fish in each channel (see Figure 1 in Olsén et al., 1998). M*Rv* is a measure of preference and a positive M*Rv* means that the fish is attracted to water from aquarium 1 that contained a fish with the same MHC genotype as the test fish. Experiment 1: water scented by MHC-identical siblings that were MHC-different or similar to the test fish (control test); experiment 2: "Full sibs, same MHC vs. full sibs, different MHC"; experiment 3: "non-sibs, same MHC vs. full-sibs, different MHC." The results of the different experiments were compared with repeated measures ANOVA followed by Tukey-Kramer multiple comparison test. Student's *t* test ($t = \mathrm{M}Rv/\mathrm{SD}/\sqrt{N}$) was used to compare the M*Rv* for each experiment with the theoretically indifferent reaction when $\mathrm{M}Rv = 0$. $\mathrm{M}Rv = 0$ when the number of observations in each water quality is the same. $^{*}P < 0.05$.

control tests and when the fish chose between siblings of different MHC genotype ($q = 4.41$, $P < 0.05$). The Student's *t* test showed that only in experiment 2 was the M*Rv* different from the theoretically indifferent reaction when M*Rv* = 0 ($t_4 = 2.56$, $P < 0.05$, one tailed). The results show that the test fish preferred water scented by an MHC-identical sibling, but no significant preference was observed during a choice between an MHC-like nonsibling and a sibling that was different. Test fish that had been isolated since fertilization showed no significant preference between water scented by MHC-like and nonlike siblings (Figure 2).

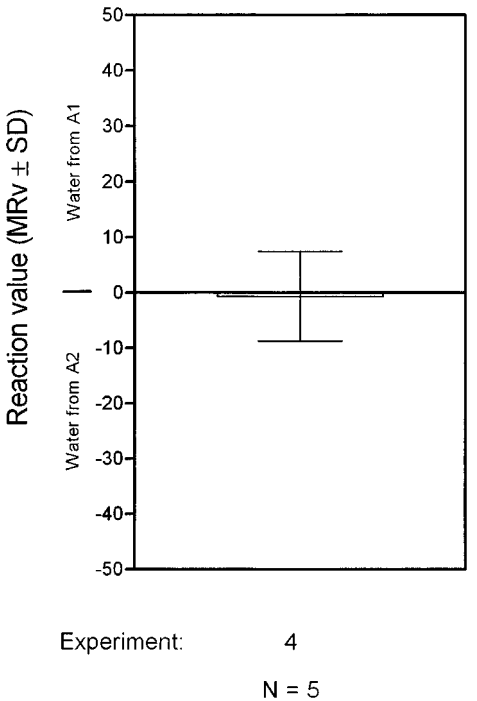


FIG. 2. In experiment 4, the test fish had been isolated since fertilization. An MHC-identical sibling scented one side of the test area and an MHC-different sibling scented the other side. For further details see Figure 1.

DISCUSSION

In the present study, we have demonstrated that juvenile Arctic char are not able (or not sufficiently motivated) to discriminate between odors from a sibling and a nonsibling when the sibling donor has a different MHC class II genotype compared to the test fish and where the nonsibling donor was identical. We also observed, as in a previous study (Olsén et al., 1998), that in tests using only siblings, the test fish prefer to be in water scented by an MHC class II-identical donor. The previous study showed also that a nonsibling with one MHC allele in common with the test fish gave no significant preference, but attraction to sibling-scented water was observed when both donors were different from the test fish but had one allele in common with each other. The results suggest that the test fish is probably guided by both MHC class II-linked odors plus odors dependent on other genes. If only MHC class II was responsible for the odors, the test fish should have chosen the water scented by the MHC-like nonsibling. The test fish had problems in choosing,

which led to an indifferent response. A likely candidate for this influence is genes in the MHC class I region, which, unlike all other vertebrates, is unlinked to the class II region in teleost fish (Sato et al., 2000). Class I genes encode membrane proteins on the outside of all cells (transplantation antigenes), and class II genes are responsible for the production of the polypeptides on the surface of B lymphocytes and macrophages that are important for the process of self-recognition capacity in other lymphocytes (Klein, 1986; Gardner et al., 1991). If the test fish used overall genetic similarity as a cue, it should have preferred the water scented by the sibling donor in all situations. This was not the case. Experiments with rodents have also shown that both MHC and other genes may have an influence on the animals' individual odors, which can be used as information about kinship (Lennington et al., 1988; Brown et al., 1989; Boyse et al., 1991; Schellink et al., 1993). Learning, as demonstrated by foster nursing, is also an important part of the mechanism (e.g., Boyse et al., 1991; Penn and Potts, 1998a). The results with isolated char also suggest that the ability to discriminate between odors from MHC class II-identical and nonlike siblings is learned. However, we can not rule out the possibility that the fish recognized the differences, but were not motivated enough to demonstrate a behavior discrimination. We know from previous studies that young Arctic char learn the odors used in sibling discrimination, and that social interactions are important and result in a more permanent ability to discriminate. (Winberg and Olsén, 1992; Olsén and Winberg, 1996).

The next step in the present study of behavioral discrimination of juvenile Arctic char will be to investigate the importance of the class I MHC regions. Furthermore, it is also of interest to analyze the significance of the commensal microflora on the odors released and detected by conspecifics. In rats, the odors of individuality have been shown to be dependent on commensal bacteria (Singh et al., 1990), and a recent study has shown that fish emit odors that are dependent on an intact bacterial flora (Ringelberg and van Gool, 1998).

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CHEMICALS OF PREDATORY MOSQUITOFISH (*Gambusia affinis*) INFLUENCE SELECTION OF OVIPOSITION SITE BY *Culex* MOSQUITOES

KIM A. ANGELON and JAMES W. PETRANKA*

Department of Biology
University of North Carolina at Asheville
Asheville, North Carolina 28804

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Abstract—Ovipositing insects may avoid aquatic sites where there is high predation risk to their offspring, but the proximate mechanisms that mediate avoidance behavior are poorly resolved. We conducted an experiment to determine whether mosquitoes would reduce oviposition rates in pools containing chemicals of the mosquitofish (*Gambusia affinis*), a voracious predator that is widely employed to control mosquitoes. Experimental treatments consisted of outdoor pools that contained known concentrations of fish chemicals (low, medium, or high) or no fish chemicals (control). The pools were arranged in a randomized block design, and the number of mosquito larvae in each pool served as the response variable to estimate relative oviposition rate. Members of the *Culex pipiens* complex were the main colonizers of the pools. The mean number of larvae per pool differed among treatments ($P = 0.026$) and was about three times greater in control pools compared with those receiving medium and high concentrations of fish chemicals. Pairwise comparisons indicate that only medium and high treatments differed significantly from controls, suggesting that a threshold concentration exists below which mosquitoes cannot reliably detect predators. Our data suggest that the effectiveness of *Gambusia affinis* in controlling mosquitoes may be compromised if adult mosquitoes respond to fish stocking by shifting to nearby breeding sites that lack fish. We discuss issues concerning the use of *Gambusia* in biological control programs within the context of these new findings.

Key Words—Chemical cues, predation, mosquitoes, *Culex pipiens*, *Culex quinquefasciatus*, fish, *Gambusia affinis*, oviposition, predator avoidance, predator detection.

* To whom correspondence should be addressed. E-mail: petranka@unca.edu

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INTRODUCTION

Chemically mediated avoidance is an adaptation used by prey to detect and evade predators. Weldon (1990), Chivers and Smith (1998), and Kats and Dill (1998) reviewed studies showing that fish, amphibians, reptiles, mammals, a bird, and a broad array of invertebrates have evolved chemosensory mechanisms for detecting predators. Common responses to predator chemicals include increased refuge use (e.g., Kats et al., 1988), marked changes in rate of movement (e.g., Williams and Moore, 1985; Mathis et al., 1993; Ode and Wissinger, 1993; Chivers et al., 1996; Huryn and Chivers, 1999), reduced foraging (e.g., Petranka and Hayes, 1998), reduced courtship behavior (Stein, 1979), and predator avoidance (Flowers and Graves, 1997). Prey respond more strongly to sympatric predators than allopatric predators (Mathis et al., 1993) and when natural exposure to a predator is high versus low (Kats et al., 1988). These findings suggest that natural selection may adapt prey to local predator regimes.

Kats and Dill (1998) list only 16 studies that provided evidence for chemically mediated detection of predators by insects, and almost all involve the larvae of aquatic species responding to aquatic predators. Mechanisms for predator detection by insects may involve tactile, visual, or chemical cues. Where chemical cues are involved, responses to predators are often influenced by the predator's diet. For example, mayfly and damselfly larvae are more likely to respond to fish chemicals if the fish recently consumed conspecific prey (Chivers et al., 1996; Huryn and Chivers, 1999).

Many aquatic insects have complex life cycles that include a relatively sedentary, aquatic larval stage and a volant adult stage. While searching for oviposition sites, insects may encounter an array of microhabitats or isolated habitats (e.g., individual ponds) that vary with respect to future offspring survival. Components of habitat quality that may influence offspring fitness are the density of competitors, the risk of predation, and the seasonal duration and productivity of the habitat.

Selection should favor individuals that can assess habitat quality and choose microhabitats for ovipositing that maximize offspring survival. The few studies that have addressed this question indicate that ovipositing mosquitoes and phantom midges will avoid pools containing egg or larval predators such as notonectids, odonates, tadpoles, and fish (Chesson, 1984; Petranka and Fakhoury, 1991; Blaustein and Kotler, 1993; Ritchie and Laidlaw-Bell, 1994; Stav et al., 1999). However, the proximate cues by which ovipositing insects detect predators are poorly resolved.

Petranka and Fakoury (1991) demonstrated that mosquitoes and phantom midges greatly reduced ovipositing rates in experimental pools that contained caged sunfish (*Lepomis*) that were not visible to ovipositing females. The mechanism by which mosquitoes detect fish was not determined, but chemosensory detection seemed most likely based on the experimental design. Here, we examine

whether ovipositing mosquitoes can detect the presence of a predatory fish (*Gambusia affinis*) using only chemical cues. We also examined whether female mosquitoes can use the relative concentration of predator chemicals to gauge predation risk.

METHODS AND MATERIALS

We collected mosquitofish (*Gambusia affinis*) from a pond in Mecklenburg County, North Carolina, to condition water with predator chemicals. We used three sets of plastic pools (1.3 m diameter; 0.15 m deep) for fish husbandry and water conditioning. These included a set of maintenance pools for feeding and raising fish, a set of clearing pools to allow fish to empty their guts, and a set of conditioning pools to condition the test water with fish chemicals. Pools were placed near the partially shaded edge of a field in Asheville, North Carolina, and were exposed to natural temperature fluctuations. Fish were fed and allowed to reproduce in maintenance pools that contained dechlorinated water, leaf litter, and *Elodea densa*, which provided cover and oxygenated the water. We added aquarium salts and adjusted the pH to slightly above seven with baking soda to reduce physiological stress on the fish.

We maintained fish on a regular feeding schedule of tropical fish food flakes. We did not use natural prey because many organisms use chemicals from injured conspecifics to detect predators (e.g., Chivers et al., 1996; Laurila et al., 1997; Chivers and Smith, 1998; Huryn and Chivers, 1999), and we did not want to confound the effects of predator chemicals with those from injured prey. Newborns were separated from adults to prevent cannibalism (Lloyd et al., 1986).

We began adding fish chemicals to test pools (see below) on July 7, 2000, and the experiment ran for 18 days. Prior to conditioning water that was added to test pools, we transferred fish into clearing pools containing an equal mixture of dechlorinated tap water and water filtered from other holding pools. We allowed the fish to fast and clear their guts for one day before being transferred to the conditioning pools.

We conditioned water with fish chemicals by maintaining 10 adults and 20 juvenile fish in a wading pool that contained only aged tap water and aquarium salts. We replaced 10 fish in the conditioning pools daily with fish taken from the clearing pools. These were returned to the maintenance pools for feeding and rotated through the clearing and conditioning pools as the experiment progressed. Water that was withdrawn to add to test pools was replaced daily with dechlorinated water (about 25% of the total volume) and replacement water was conditioned with fish chemicals for about 12 hr before being added to test pools.

We used a randomized block design to examine the response of mosquitoes to four experimental treatments (see below): a control that lacked fish chemicals, and low, medium, and high levels of water conditioned with fish. We set up 40 plastic

pools (1.0 × 0.2 m) in two rows in a partially shaded area of a field within 3 m of tree cover. There were 10 blocks (replicates), and the four experimental treatments were randomly assigned within each block. Each pool was filled to a depth of 10 cm, and 20 g of leaf litter was added to provide nutrients for larval growth and to attract ovipositing mosquitoes. A dead branch was placed on the side of each pool as a perch site for the females. Pools were seeded with invertebrates and microorganisms by adding 0.5 liters of water from a local vernal pond. Water from the pond was placed in a large bucket, mixed, and haphazardly assigned in 0.5-liter increments to the pools. These conditions provided an array of natural background chemicals and organisms to increase realism.

The treatments consisted of adding 2 liters of water twice daily that contained different ratios of conditioned and unconditioned water. The respective volumes (in liters) of unconditioned and conditioned water for treatments were 2:0 in control, 1.5:0.5 at low concentration, 1:1 at medium concentration, and 0:2 at high concentration. Conditioned water was taken from the conditioning pool containing 30 unfed *Gambusia affinis*, while unconditioned water was from similar pools that lacked fish and contained only dechlorinated water. Because fish chemicals will decompose with time through bacterial decay, we added 2 liters of water each day in early morning and late afternoon. The estimated dilution ratio for the high treatment was 20:1 (ratio of volume of water in pools/total volume of conditioned water added daily).

The aquatic phase of the mosquito life cycle includes four larval instars followed by a pupal molt (Gillett, 1972). Because mosquito eggs are difficult to locate in pools, we used the number of mosquito larvae as an estimate of the number of eggs laid. We terminated the experiment after the first pupa was sighted and two weeks after the first sighting of mosquito larvae. We retrieved larvae from pools by filtering out coarse debris and then filtering all water through fine-mesh plankton nets. We preserved samples in 70% ethanol and removed and counted all mosquito larvae using dissecting microscopes. We identified the fourth-instar larvae to genus and species complex.

We analyzed data by using Statistical Analysis System (SAS Institute, 1989). Preliminary analyses revealed that the assumptions of normality and homogeneity of variances were violated and could not be corrected using data transformations. We subsequently used ranked data in the analysis (PROC rank of SAS) with a general linear model. The Ryan-Einot-Gabriel-Welsch multiple range test was used for pairwise comparison of the means. The REGW test controls for a type I experimental error.

RESULTS

Early-instar larvae could not be identified, but all fourth-instar larvae identified were members of the *Culex pipiens* complex. Two sibling species (*C. pipiens*

and *C. quinquefasciatus*) occur in western North Carolina and cannot be separated by larval characteristics (King et al., 1944). Because our pools may have contained both species, we refer to all larval forms as *Culex* spp.

The mean number of *Culex* larvae per pool was highest in the control ($\bar{X} = 27.2$) and low ($\bar{X} = 38.1$) treatments and markedly lower in the medium ($\bar{X} = 8.4$) and high ($\bar{X} = 9.2$) treatments (Figure 1A). The high mean and SE for the low treatment was due to one extreme value ($N = 300$ larvae), with the second highest value for any pool being 77 larvae. Means for ranks, which better reflect central tendency, progressively decreased with the relative concentration of fish chemicals (Figure 1B).

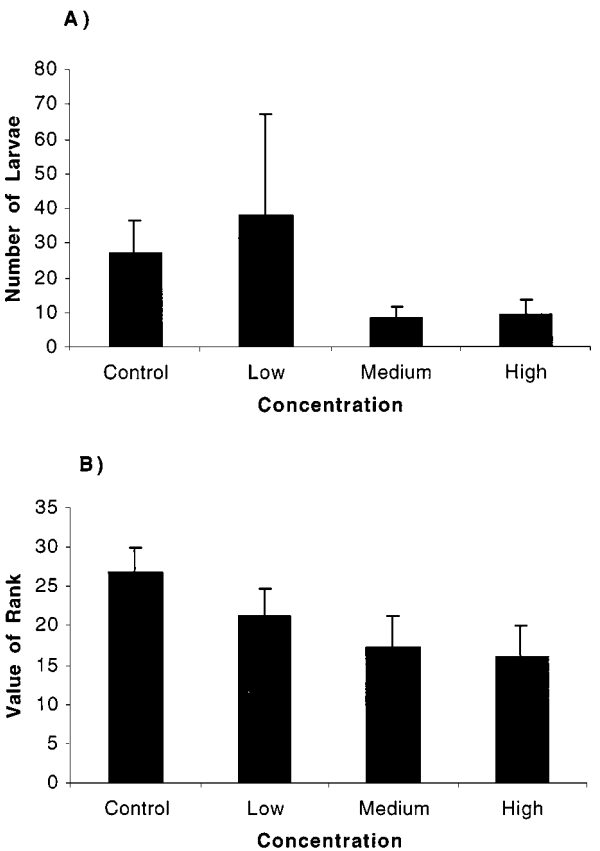


FIG. 1. Response of ovipositing mosquitoes to low, medium, or high concentrations of *Gambusia affinis* chemicals. Controls lacked fish chemicals. Bars are means +1 SE. (A) Graph is based on raw data, and (B) on ranked values.

TABLE 1. SUMMARY STATISTICS OF ANOVA FOR EFFECTS OF TREATMENTS ON NUMBER OF MOSQUITO LARVAE IN POOLS[a]

Source	*df*	Mean square	*F*	*P*
Blocks	9	313.75	4.86	0.0006
Treatments	3	232.18	3.60	0.026
Error	27	64.52		
Total	39			

[a] The analysis was conducted using ranked values. Blocks correspond to replicates.

ANOVA revealed a significant block effect ($P = 0.0006$) and significant treatment effect after the block effect was removed ($P = 0.026$; Table 1). The significant block effect was due to higher numbers of larvae in replicates that were near a shaded building that may have served as a diurnal resting site for mosquitoes. Pairwise comparisons using the REGWQ test revealed that the means for the control and low treatments did not differ significantly; however, means for medium and high concentrations differed significantly from controls.

DISCUSSION

This research is the first to demonstrate that ovipositing insects can use chemosensory mechanisms to assess predator risk to offspring. Grostal and Dicke (1999) recently demonstrated similar behavior in acarine mites, suggesting that the phenomenon may occur across a diverse array of arthropods. Kairomones or semiochemicals emitted from predators (sensu Nordlund, 1981) normally are used by prey to minimize their encounters with dangerous community associates (Kerfoot and Sih, 1987; Kats and Dill, 1998). This study extends this phenomenon by demonstrating that ovipositing females may use kairomones to make behavioral decisions that affect encounter rates of future offspring with predators. Statistically, our data only show evidence of a threshold response. However, the consistent decline in rank means with relative chemical concentration suggest that *Culex* females may be capable of making graded responses to predation risk. Dilution ratios of unconditioned water to conditioned water were as high as 80:1 in our experiment. The use of a wider range of concentrations in future studies may reveal statistically significant evidence of a graded response.

Prey species are more likely to evolve chemically mediated avoidance of predators when visual detection of predators is limited (Kats and Dill, 1998; Petranka and Hayes, 1998). In addition, prey must possess suitable behaviors and chemosensory mechanisms to detect and respond to predators. Mosquitoes appear to meet all of these requirements since many species are crepuscular or

nocturnal and rely heavily on chemosensory mechanisms when selecting oviposition sites. After mating and taking a blood meal, a hemolymph-borne hormone triggers the female to start searching for a suitable oviposition site and to become receptive to chemical signals from the site (Klowden and Blackmer, 1987). Females oviposit on mud or the water's surface (e.g., *Culex* spp.) after sampling the substrate for chemical composition using receptors on the tarsi, antennae, and tip of the proboscis (Davis, 1976; Bentley and Day, 1989).

Evidence now suggests that mosquitoes use chemosensory information to assess several parameters that reflect habitat quality for offspring, including the availability of nutrients, presence of competitors and predators, and the overall quality and permanence of the water (Davis, 1976; Angerilli, 1980; Chesson, 1984; Bentley and Day, 1989; Petranka and Fakhoury, 1991; Blaustein and Kotler, 1993; Edgerly et al., 1998). The neural mechanisms by which these are integrated to make appropriate behavioral decisions are unknown, as is the extent to which individuals can balance conflicting signals.

Chemically mediated avoidance of fish has been demonstrated for a taxonomically diverse group of prey (Kats and Dill, 1998), but the kairomones involved in avoidance have not been identified or characterized. Clearly, this remains a major challenge for fully understanding predator-specific responses and evolutionary interactions between predator and prey. Identifying kairomones associated with fish may prove to be particularly challenging because these may be omitted from the fish or from members of the microflora that inhabit the slime that covers the body.

Although we were unable to quantify chemical concentrations, the small volumes of conditioned water that we added to pools daily resulted in concentrations of chemicals that were presumably well within the range that occurs in nature. *Gambusia affinis* inhabits sluggish habitats such as swamps and roadside ditches, and forms schools that may contain hundreds of individuals. Schooling behavior produces a patchy environment in which predation risk may vary locally depending on the location of schools. Female mosquitoes could avoid *Gambusia* at several spatial scales. For example, females could use the strength of fish chemicals to avoid local sites in ponds with dense schools. Alternatively, females might strongly avoid ponds with *Gambusia* and seek out ponds that lack fish (Ritchie and Laidlaw-Bell, 1994).

Researchers have questioned the use of mosquitofish as biological controls, especially when introduced as exotics to supplement native species. Moyle (1976) showed that mosquitofish introduced in 1922 into southern California subsequently reduced populations of native fish throughout the state due to competition, predation, and hybridization. *Gambusia affinis* preys on amphibian eggs and larvae and could be contributing to certain anuran declines (Grubb, 1972; Gamradt and Kats, 1996; Komak and Crossland, 2000). Despite its reputation as being an effective predator on mosquitoes, Courtenay and Meffe (1989) concluded that *Gambusia* is generally ineffective as a biological control agent. For example, *Gambusia* had

a positive impact on controlling mosquitoes in only four of 20 countries where it was introduced for mosquito control or other reasons.

Many mosquitoes such as *Culex pipiens* and *C. quinquefasciatus* breed in small, fish-free habitats such as artificial containers (King et al., 1944), a pattern that may reflect adult choice more so than exclusion from fish ponds because of direct predation (lethal effects). Thus, one explanation for the ineffectiveness of *Gambusia* in controlling mosquitoes is that ovipositing mosquitoes may seek out fish-free habitats that adjoin large bodies of water containing *Gambusia*. The active avoidance of habitats with high predation risk to offspring may ultimately act to sustain high densities of mosquitoes near areas stocked with *Gambusia*. This hypothesis is supported by Ritchie and Laidlaw-Bell (1994), who found that ovipositing *Aedes taeniorhychus* strongly avoided sites with high densities of *Gambusia holbrooki* and shifted to adjoining habitats with few or no predatory fish.

We encourage research to more carefully examine the importance of nonlethal effects and adult choice of oviposition sites when designing mosquito control programs involving predators. We also encourage researchers to consider both evolutionary and ecological interactions between predator and prey when designing biological control programs.

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INTERNET PROGRAMS FOR DRAWING MOTH PHEROMONE ANALOGS AND SEARCHING LITERATURE DATABASE

JOHN A. BYERS*,†

Department of Crop Science
Swedish University of Agricultural Sciences
SE-230 53 Alnarp, Sweden

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Abstract—An Internet web page is described for organizing and analyzing information about lepidopteran sex pheromone components. Hypertext markup language (HTML) with JavaScript program code is used to draw moth pheromone analogs by combining GIF bitmap images for viewing by web browsers such as Netscape or Microsoft Internet Explorer. Straight-chain hydrocarbons of 5–22 carbons with epoxides or unsaturated positions of *E* or *Z* geometrical configuration with several alternative functional groups can be drawn by simply checking menu bars or checkboxes representing chain length, *E*/*Z* unsaturation points, epoxide position and chirality, and optional functional groups. The functional group can be an aldehyde, alcohol, or ester of formate, acetate, propionate, or butyrate. The program is capable of drawing several million structures and naming them [e.g., (*E*,*E*)-8,10-dodecadien-1-ol and abbreviated as *E*8*E*10–12:OH]. A Java applet program run from the same page searches for the presently drawn structure in an internal database compiled from the Pherolist, and if the component is found, provides a textarea display of the families and species using the component. Links are automatically specified for drawn components if found in the Pherolist web site (maintained by H. Arn). Windowed links can also be made to two other JavaScript programs that allow searches of a web site database with over 5900 research citations on lepidopteran semiochemicals and a calculator of vapor pressures of some moth sex pheromone analogs at a specified temperature. Various evolutionary and biosynthetic aspects are discussed in regard to the diversity of moth sex pheromone components.

*E-mail: jbyers@wcrl.ars.usda.gov
† Present address: Western Cotton Research Lab, USDA-ARS 4135 East Broadway Rd., Phoenix, Arizona 85040-8830.

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Key Words—Lepidoptera, moth pheromone components, epoxides, aldehydes, alcohols, acetate esters, Internet, JavaScript, computer program, Java applet, database, vapor pressures.

INTRODUCTION

During the last few years, the Internet has become accessible to most scientists throughout the world. The quantity of information available through the Internet, or World Wide Web, is likely to grow, improve in quality, and become ever more important to research productivity and education (Byers, 1999; Zenger and Walker, 2000). Web pages are composed of hypertext markup language (HTML) code and are interpreted by web browsers such as Netscape Communicator and Microsoft Internet Explorer (IE), accounting for 37% and 61%, respectively, of 9394 visitors to the menu page, www.vsv.slu.se/cec/h.htm (during 2000, eXTReMe Tracking). JavaScript is a language, originating from Netscape Comm. Corp., that can be loaded from a web page and run by browsers to achieve a moderate level of interactivity and data processing (Goodman, 1996). Java applets (Sun Microsystems Inc.) are separately compiled programs that are called into action by the HTML or JavaScript code and run by the browser or associated software to yield a true program environment, although with security constraints so that only the screen image can be manipulated (Lemay and Perkins, 1997; Vanhelsuwé et al., 1997; Cadenhead, 1999). The use of java applet programs makes it possible to achieve full interactivity and advanced graphical applications. However, by clever use of bitmapped images, JavaScript can also produce interesting visual effects.

From 1975 to 1991 in the *Journal of Chemical Ecology*, there were 619 articles published on Lepidoptera, which accounted for 29% of the journal's reports (Byers, 1992). During the subsequent nine years, 1992–2000, this figure dropped slightly to 25% of 1840 articles. For all insect pheromone papers (1993–2000), BIOSIS Previews cites 3775 articles with 45.3% on Lepidoptera (nearly all on moths). Due to this large interest in lepidopteran pheromones, it was desirable to make a web page that draws and names most moth pheromone components and analogs by simply selecting checkboxes or buttons controlled by JavaScript code. I also wanted the web page to run a Java applet that would immediately tell the user if the drawn compound was a known pheromone component of one or more species in the Pherolist (Arn et al., 1992; Arn, 2000), the well-known web site covering moth pheromone components (www-pherolist.slu.se/index.html). If a known component is drawn, then the page should allow the user to save time by linking automatically to the specific component in the Pherolist. Another goal was to link the page to a searchable web database on moth literature citations (Byers, 2000) and a calculator of moth analog vapor pressures. The final objective was to discuss the diversity of double bond positions of moth sex pheromone components (Pherolist) and analogs

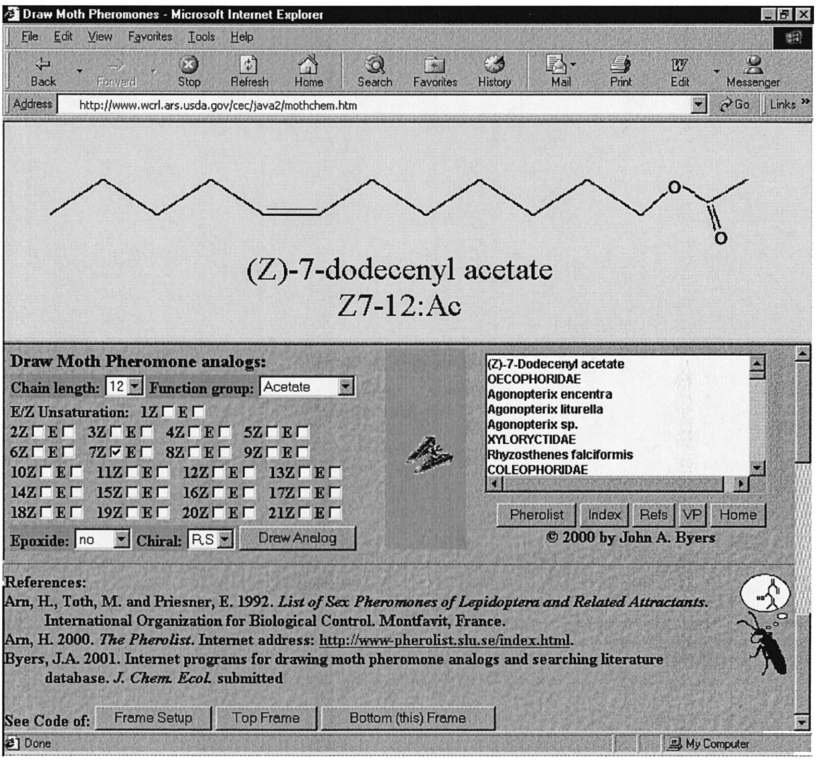


FIG. 1. Internet browser view of frames of web page that draws moth pheromone analogs in top frame based on user input in the bottom frame. Functions of various buttons and textboxes are explained in the text.

(program drawn) in regard to evolutionary and biosynthetic aspects. The web page is at the Internet address: www.wcrl.ars.usda.gov/cec/java2/mothchem.htm (Figure 1).

METHODS AND MATERIALS

The JavaScript code for drawing moth pheromone analogs is found in web frames that can be viewed by Netscape and IE by right-clicking on the frame and selecting "view source." Alternatively, the code for each frame can be viewed from pop-up windows (three buttons at bottom of lower frame, Figure 1).

To draw a molecule, the user selects a chain length from 5 to 22 from a drop-down menu with only one option selection allowed (Figure 1, lower frame). The

same input method is used for selecting the epoxide position and chirality as well as the functional group: either none, an aldehyde, alcohol, or esters of formate, acetate, propionate, and butyrate. A pair of checkboxes for each possible carbon position in the chain can be checked to designate geometrical configuration (*E* or *Z*) of double bonds. Only one box of a pair can be checked. After input is selected, the "draw analog" button is clicked. Several possible error messages may occur if an *E*/*Z* or epoxide position checkbox is selected outside the chain length or is next to position 1 of a functional group. Furthermore, adjacent positions cannot both be unsaturated nor can they be next to or within an epoxide, as these structures are chemically unlikely. Chains with unsaturation at the end opposite the functional group cannot be designated *E* or *Z* (but are delta).

The program then takes the input data and converts them to variables whose values are used to control the drawing of a molecule in the upper frame (Figure 1). The drawing is composed of an appropriate permutation of 22 bitmapped GIF images with transparent backgrounds. These are scaled according to the viewer's screen resolution and displayed from left to right such that the molecule is drawn from the highest numbered carbon toward the functional group. Six of the GIF images represent carbon–carbon bonds, 6 are for epoxides, and 10 are for different configurations of the 6 possible functional groups. The variables are also used to construct the IUPAC name, e.g., (*E*,*E*)-8,10-dodecadien-1-ol, and a short name, *E*8*E*10–12:OH. A further abbreviated name, e.g., e8e1012oh, is made and searched for in an internal database array of about 420 names. If found, as it would be in this case (codlemone), the abbreviation serves as the prefix of the HTML file name in the Pherolist in order to allow automatic linking to this file. The 420-name internal database was made by a BASIC program that extracted pheromone components (and female-produced analogs that could be drawn) from the 544 HTML files (1.5 MB) concerning components that are in the Pherolist as of June 2000.

Another feature found at the right of the lower frame (Figure 1) is a Java applet that is represented by a scrollable text area. The applet consists of a database of component names and the corresponding insect families and species that have or are attracted to the components as extracted from the Pherolist by the BASIC program. Initially the text area informs the user of the purpose of the text area and gives credits to the Pherolist. The data of the applet consists of an array of nonredundant names of families and species in no particular order, but simply as they were encountered in the 544 HTML Pherolist files on chemicals. Each of these files has the component name, as found between the beginning and ending HTML tags of the title, e.g., <TITLE>(E,E)-8,10-dodecadien-1-ol</TITLE>. Similarly, there are unique text strings that indicate the families, genera, and species reported to be attracted to or produce the component. However, the resulting extracted file was larger than 64 KB, the maximum size for a method in Java (version 1.2.2), so the information was indexed using numbers for the array of nonredundant

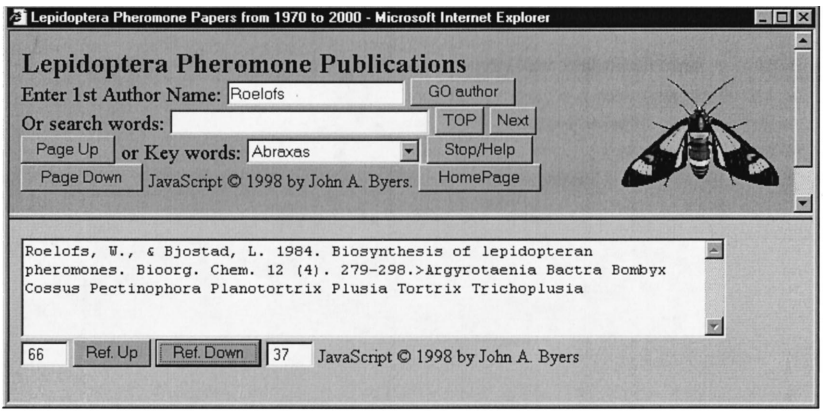


FIG. 2. Internet browser view of frames of a JavaScript database show a search for a specific citation from among currently 5912 references on semiochemicals of Lepidoptera.

species/families mentioned above. This allowed 420 components to be compared to 1716 species and families in the Java applet. Thus, when the user draws a component, e.g., (*E*,*E*)-8,10-dodecadien-1-ol, the Java applet searches its database and shows the names of all families and species attracted by this component (e.g., three subfamilies of Tortricidae and 13 species).

Another feature is a button ("Refs") that, when clicked, allows the user to open a pop-up window with two frames of a web database of citations on over 5900 papers of lepidopteran semiochemicals (Figure 2). A BASIC program similar to that reported earlier (Byers, 1992) reformatted the database, which served as input to another BASIC program that fused templet files of HTML and JavaScript code with the reformatted data to produce 92 web pages with self-searching capability by using JavaScript code and "cookies." Cookies are browser files maintained on the user's hard drive that store information that normally would vanish when the user moved to another web page. None of the literature web pages has more than 30 KB of data since it was found that data strings larger than 32 KB caused browsers to incompletely load the page. To update the list of citations, the entire database must be reformatted by the BASIC program into 30-KB blocks such that no first author is split between pages. The BASIC applications were programmed with Microsoft QuickBASIC version 4.5, while the Java applet (small application) was done with the Sun Microsystem' 1.2.2 package.

Still another feature is a button ("VP") that displays a window with a JavaScript coded calculator of vapor pressures of a few unsaturated acetate esters as well as saturated aldehydes, alcohols, hydrocarbons, and fatty acids with 10 or more carbon chain lengths (Figure 3). A compound is selected from a pop-up menu on the left and a temperature is entered on the right so that the vapor

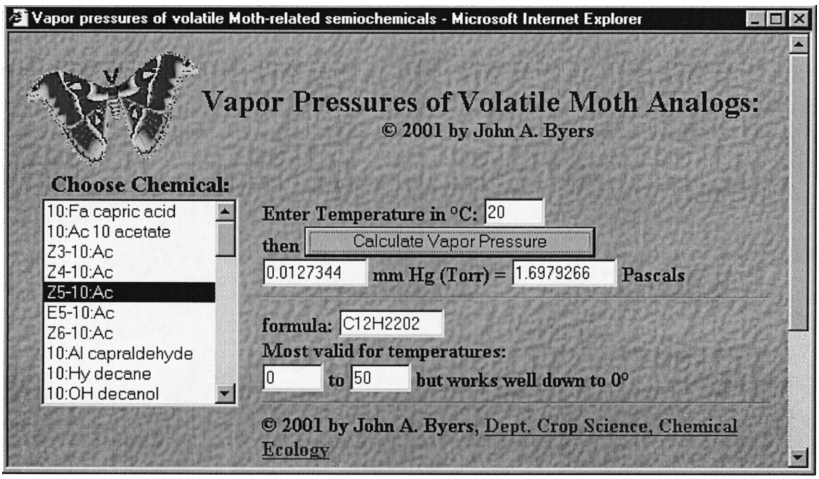


FIG. 3. Internet browser view of a pop-up window of a JavaScript web page for calculating vapor pressures of some moth pheromone components and fatty acids at different temperatures.

pressure (V) can be calculated from the equation, $V = 10^{(-0.2185\,A/K)+B}$, where V is in millimeters of mercury (torrs), A is molar heat of vaporization in calories per gram mole, K is temperature in degrees Kelvin, and B is a constant (Schlessinger, 1971). Exponential regression was used on the data of Olsson et al. (1983) to obtain vapor pressure equations for some moth acetate esters. Vapor pressures can also be estimated from boiling points at partial pressures according to the methods of Hass and Newton (1971).

RESULTS AND DISCUSSION

Operation of the web page for drawing unsaturated straight-chain hydrocarbons with various functional groups is user-friendly. Only two drop-down menu bars and possibly a few checkboxes need to be selected to draw and name the majority of natural moth pheromone components (Figure 1). The program can draw the 43 unbranched hydrocarbons with no functional group, 71 epoxides, 82 aldehydes, 66 alcohols, 167 acetate esters, and 10 other esters listed in the Pherolist. Thus, about 83% of the 528 currently listed components (produced or attractive) in the Pherolist can be drawn (16 additional analogs containing chlorine, fluorine, or nitrogen are considered unnatural). During the extraction of compound names from the Pherolist, names with "methyl," "chlor," "fluor," "one," "oate," "yn," "2-ol," "iso," "nitr," and "valer" were excluded to simplify the drawing function. Of the

71 epoxides known, all but one have one epoxide per compound and these are all *cis*-configured, being either of two chiral forms, *R,S* and *S,R*, so it was possible to include these components (Millar, 2000; Pherolist). However, the chiralities of about half of the aliphatic epoxides (all *cis*) have not been determined. Although chemists have made *trans*-configured epoxides, they have not been discovered in moths (Millar, 2000; Pherolist). One important group that was excluded contains 48 methyl-branched analogs. This group was not considered since the structures are often chiral and, thus, naming becomes more complex. The remaining 49 excluded names were: "one" (16), "oate" (13), "2-ol" (8), "iso" (6), and "valer" (1).

The program also can draw several million unused or undiscovered analogs. For example, considering 14-carbon acetate esters, there are 23 different singly unsaturated compounds, 200 doubly unsaturated ones, and 816 triply unsaturated ones (*E*, *Z*, or delta) (Byers, unpublished). Even more compounds are possible when four (1568), five (1232), and the maximum of six unsaturations (256) are considered. However, further unsaturation in a molecule would make it less stable due to chemical alteration by hydrogenation or geometric isomerization. Natural selection would not favor the evolution of a less stable pheromone component that otherwise would require continuous production of large quantities to be effective. Naturally occurring longer chain hydrocarbons have been reported with up to three unsaturations and rarely four, e.g., (*Z,Z,Z,Z*)-3,6,9,11-nonadecatetraene in *Alsophila pometaria* (Geometridae) (Wong et al., 1984) and (*Z,Z,Z,Z*)-7,13, 16,19-docosatetraen-1-ol isobutyrate in *Euproctis chrysorrhoea* (Lymantiidae) (Leonhardt et al., 1991), but none have been found with five. Theoretically, 10 unsaturations can fit within a 22-carbon chain acetate ester and be named by using the program. An analysis of the 294 unbranched naturally occurring aldehydes, acetate esters, and alcohols (Pherolist) shows that many possible permutations of unsaturations have not been reported in the Lepidoptera. There were 125 components with one unsaturation (42%) and 130 components with two unsaturations (44%). Of the latter, 77% have one single bond in between the unsaturations (conjugated), while 5% have two single bonds between the unsaturations, and 18% have more than two single bonds between. Only 16 components (5%) have three unsaturations and none have four unsaturations (only a few straight chain hydrocarbons have four).

The number of different components reported in moths to date (about 528) appears strikingly few when compared to the several million unsaturated *E* and *Z* analogs potentially available as sex pheromone components. For example, there are 1039 possible 14-carbon chain acetate ester components with one, two, or three unsaturations (*E*, *Z*, or delta) (Byers, unpublished data), but only 42 of these have been identified in moths (Pherolist; Byers, unpublished data). The limitation to essentially 1039 possible analogs may indicate this is a sufficiently large number to allow any species to evolve a unique blend from among these. However, there may not have been enough evolutionary time for speciation events to create more

divergence and use of more structures. The limitation to three or less unsaturations in components may also be due to chemical constraints such as increased instability with desaturation.

The enzyme systems for desaturation and chain-shortening involved in moth component biosynthesis (Bjostad et al., 1981; Roelofs and Wolf, 1988; Tillman et al., 1999) may have evolved in response to the inherent stability of mono- and di-unsaturated hydrocarbons as compared to less stable multiunsaturated alternatives. Probably the most obvious hypothesis to account for the relatively few unsaturations in a moth component is that the fatty acid precursors (oleic, linoleic, and linolenic acids) have one, two, or three unsaturations, respectively. However, as mentioned above, a few moths biosynthesize aliphatic chains with four unsaturations, and many odd- and even-numbered carbon bond positions can be unsaturated by the Δ5, 9, 10, 11, 12, and 14 desaturases and chain-shortening enzymes that have been discovered so far (Bjostad et al., 1981; Roelofs and Wolf, 1988; Foster and Roelofs, 1988, 1996; Zhao et al., 1990; Jurenka, 1997; Tillman et al., 1999). If there are constraints on the evolution of biosynthetic enzymes, there could also be limits on the ability of antennal receptors to discriminate among higher numbers of unsaturated bonds (and constraints on the evolution of more complexity).

The limited numbers of unsaturated positions in moth analogs might also be due to some disadvantage in using molecules with increased volatility due to higher numbers of unsaturated bonds. The double bond length between carbons is shorter than in a single bond (also the H—C bonds are shorter on double bond carbons), which makes the molecule smaller in area and, thus, more volatile (Morrison and Boyd, 1973). There are few data on vapor pressures of moth acetate esters with multiple unsaturations. Olsson et al. (1983) found that *Z*9–14:Ac (16 carbons) has a vapor pressure of 7*E*-4 mm Hg at 30.5°C, which is similar to 14-carbon myristic acid [3*E*-4 mm (Schlessinger, 1971)]. Thus, comparing 18-carbon fatty acids as models for 18:Ac, the differences in vapor pressures among 18-carbon fatty acids with none, one, two, or three unsaturations (stearic, oleic, linoleic, and linolenic acid) appear to be only about 2.5-fold using methods of Schlessinger (1971) and Hass and Newton (1971). Moth species have used pheromone components commonly with chain lengths from 18 down to 10, representing a decrease in volatility for saturated fatty acids of about 54-fold, for alcohols of about 33-fold, and for aldehydes of about 122-fold (Schlessinger, 1971; Hass and Newton, 1971). It seems doubtful that the modest increases in volatility by increasing desaturation would prevent evolution toward use of components with further unsaturation. However, no data were found to ascertain the effect of adding more than three double bonds on vapor pressures of moth pheromone components.

Ultimately, there is no selection pressure or fitness benefit to making a pheromone component more complex (more unsaturations or groups) once a unique species-specific structure has evolved. It is, however, apparently easier to combine two simpler components into a unique blend than to increase the

structural complexity of a single component pheromone to achieve specificity. It is expected that there would be costs to maintain unnecessarily complex systems for biosynthesis and reception when simpler ones suffice.

In moth pheromone components, two unsaturated bonds are always separated by at least one saturated bond, and position 1 is never unsaturated in aldehydes, alcohols, and acetate esters due to a maximum of four carbon bonds, keto–enol tautomerism, and dipole moments, respectively (Morrison and Boyd, 1973). Of the different moth 14-carbon acetate esters with two double bonds, the average number of saturated bonds separating two unsaturated bonds is 1.7 ± 0.66 (95% CL, $N = 20$, Pherolist). Theoretically, if two unsaturated bonds in a 14-carbon chain acetate ester are located in all possible ways such that they are separated by at least one single bond, then the average number of single bonds separating unsaturated bonds would be four (calculated from BASIC code: FOR W = 10 TO 1 STEP -1: FOR X = 1 TO W: C = C + X: D = D + 1: NEXT: NEXT: AVE = C/D). This expected average separation distance of four single bonds is much larger than the observed 1.7 bonds. Of the twenty 14:Ac components, 15 have one bond separation (conjugated), three have more than two bonds, and only one has two bonds between unsaturations (as does the fatty acid 9,12-linoleic acid).

It is also of interest to note that pheromone components and analogs have been identified from relatively few moth species (1683 species from 50 families, Pherolist). In North America alone there are over 65 moth families (Borror and DeLong, 1971), and the number of species worldwide is estimated to approach 185,000 (Holloway et al., 1987). The limited number of species with identified pheromone components is probably because most work has focused on pests, and these species are a small fraction. Secondly, once certain components are known, their subsequent discovery in other species is more likely because standards are more easily obtained and analytical methods for isolation and identification have already been developed. For example, an unknown exotic structure might not pass through the gas chromatographic columns in use, might occur in trace amounts, or have no comparable GC-MS standards (for identification) or commercial sources (for field bioassay).

The web site, linked closely with the Pherolist and reference database, should provide an educational experience for learning about moth pheromone components and aid in research. If a drawn pheromone component is on the Pherolist and the “Pherolist” button is clicked, a pop-up window links automatically to the compound’s web page in the Pherolist. Another button (“Index”) pops up a window displaying the main index of compounds of the Pherolist. As mentioned earlier, the button “Refs” opens a window of a database of over 5900 citations on lepidopteran semiochemicals, and this page links to several other citation databases on semiochemicals of Coleoptera (2256 citations), Hymenoptera (1724 citations), and other insects (2348 citations). The vapor pressure calculator (“VP” button) can aid in predicting appropriate release rates of components in the lab and field

in ratios depending on the mole percent in solution times the vapor pressure. For example, if components A:B have a mole percent ratio of 1:10 in solution and vapor pressure ratios of 1:100, then component B will have a 1000-fold higher release rate than A.

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POTENTIAL USE OF CHEMICAL CUES FOR COLONY-MATE RECOGNITION IN THE BIG BROWN BAT, *Eptesicus fuscus*

JOHANNA BLOSS,[1] TERRY E. ACREE,[2] JANELLE M. BLOSS,[3] WENDY R. HOOD,[1] and THOMAS H. KUNZ[1,*]

[1]*Department of Biology*
Boston University
Boston, Massachusetts 02215

[2]*Department of Food Science and Technology*
Cornell University—Geneva
Geneva, New York 14456

[3]*Smith College*
Northampton, Massachusetts 01063

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Abstract—Bats should benefit from recognition of their roost-mates when colonies form stable social units that persist over time. We used Y-maze experiments and gas chromatography–olfactometry (GC-O) to evaluate whether female big brown bats *Eptesicus fuscus* (Chiroptera: Vespertilionidae) use chemical cues to distinguish among conspecifics. In dual-choice Y-maze experiments, females chose the scent of another female from their own roost over a conspecific female from a different roost in a majority of trials. Analysis of total body odors using GC-O suggests that individuals from a given colony may share a more common odor signature with roost-mates than with non-roost-mate conspecifics. Using four principle components derived from 15 odor variables, discriminant function analysis correctly assigned most individuals to the correct colony.

Key Words—Big brown bat, Chiroptera, colony recognition, *Eptesicus fuscus*, female philopatry, gas chromatography–olfactometry, olfaction.

INTRODUCTION

Chemicals potentially provide discrete information about an animal's physical and social environment (Albone, 1984; Dusenbery, 1992). Olfactory cues may function at the individual and species level for the detection of predators and prey,

* To whom correspondence should be addressed. E-mail: kunz@bu.edu

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for advertisement of social and reproductive status, as territorial markers, and for kin-recognition (e.g., Eisenberg and Kleiman, 1972; Halpin, 1986; Loughry and McCracken, 1991; Ylönen, 1994; Ferkin and Johnston, 1995).

Because many bats are highly gregarious and form stable social units (Kunz, 1982; McCracken and Wilkinson, 2000), olfactory cues may convey important information about individual or group identity (Bloss, 1999). Olfaction is important in mother–infant recognition in *Tadarida brasiliensis* (Gustin and McCracken, 1987; Loughry and McCracken, 1991), *Plecotus auritus* (De Fanis and Jones, 1995a), and *Pipistrellus pipistrellus* (De Fanis and Jones, 1996). Harem-males in *Phyllostomus discolor* respond to odors from other males (Höller and Schmidt, 1993), and *Noctilio leporinus* (Brooke and Decker, 1993, 1996), *Pipistrellus pipistrellus* (De Fanis and Jones, 1995b), *Mops condylurus*, and *Chaerephon pumilus* (Bouchard, 1998) appear to use olfactory cues to recognize roost-mates.

Recent advances in the genomics of the olfactory receptor protein (OR) have revealed a large homology among mammals (Glusman et al., 2001). Given the structural and biochemical similarities in the olfactory systems of animals as diverse as humans and insects (Acree and Bloss, 1996), and the number of odors perceived by humans that induce biological activity in other organisms (Acree and Bloss, 1996), human olfaction potentially offers a valuable tool for investigating the ecology of animals that rely on chemical cues. Because humans share the same transduction system with other mammals and are likely to detect similar odors, chemicals detected by humans should include volatiles that are biologically important to both.

Olfactory-induced behavior in mammals is elicited when mixtures of chemicals reach the olfactory epithelium (Dusenbury, 1992). The present model for humans is that all chemicals in mixtures that contribute to behavior cause some perception when experienced separately (Lawless and Heymann, 1999). Although many of the chemicals detected by humans do not influence the behavior of animals, chemicals that do affect their behavior are likely to be among those detected by humans. Using gas chromatography–olfactometry (GC-O), humans can be asked about perceptions of chemicals derived from animals, although perceptions of animals cannot be measured directly without employing invasive procedures.

This study was designed to use behavioral assays to evaluate whether female big brown bats (*Eptesicus fuscus*) respond to the odors from colonymates over non-roostmate conspecifics. If females respond to olfactory cues from colony members, we predicted that these individuals should respond to the odors to which they are most familiar. We used GC-O to test for colony-specific odors. GC to separates overall body odor into its component chemicals and employes human olfaction to identify volatiles with possible odor-induced activity.

METHODS AND MATERIALS

The big brown bat, *Eptesicus fuscus* (Pallisot de Beauvois, 1796) is one of the most common and widely distributed bat species in North America (Kurta and Baker, 1990). Females form relatively stable maternity colonies that reestablish annually following hibernation and generally persist throughout the warm months. Maternity colonies are comprised primarily of females and their young and vary in size from a few dozen to several hundred individuals. Males are generally solitary and roost separate from females. In eastern North America, females usually roost in buildings and give birth to twins in mid-June. In western North America, females more commonly roost in tree cavities and give birth to singletons (Kurta, 1999).

Maternity colonies were located in southern New Hampshire (Milford) and central and eastern Massachusetts (Sherborn, Harvard, Sterling) and sampled during June and July from 1996 to 1998. Banding records were used to assess roost fidelity during the three-year study. Colonies were located in barns and consisted of approximately 30–75 adult females and their pups. Adult females were captured using either a hand net or harp trap (Kunz and Kurta, 1988) as individuals returned from their predawn foraging bouts between 04:00 and 07:00 hr. Nonvolant young were captured by hand from the maternity roost. Body mass and length of forearm were measured for each bat, and a numbered, lipped alloy band (Lambournes, Ltd., Leominster, England) was attached to the forearm of each bat for identification. Males were banded on the right forearm and females were banded on the left.

Odor samples were collected from several adult females from five different maternity roosts. Upon capture, a sterile cotton swab was rubbed 10 times over the head, chin, chest, genital area, and wings of individual females to collect odor samples. Swabs were placed in 3.5-ml amber septum vials with Teflon-lined screw caps. The tips of all swabs were bathed in 2 ml of ethyl acetate to prevent odor degradation. Four blank swabs (no odor) were treated in the same manner and used in the chemical analysis to verify that all odors were from the individual bats and not from the cotton swabs. All vials were concentrated to a volume of 0.2 ml before analysis.

We conducted behavioral tests to evaluate whether a female preferred the odor from a member of her own colony to that of a conspecific from a different colony. In a Plexiglas Y-maze (36.5-cm-long base with 31- $\times$ 6- $\times$ 6-cm arms; Figure 1), the odor swab from a colony member was placed in one arm and a swab from a noncolony member was placed in the other arm. A coin toss determined swab placement. Females to be tested were introduced individually into the base of the Y-maze, and the amount of time that each subject spent in each arm of the maze was recorded during a 5-min trial. Because only the odor swabs were presented to the females, their preferences could not be based on auditory or visual cues. Females were never tested with their own scent or tested more than twice. Between

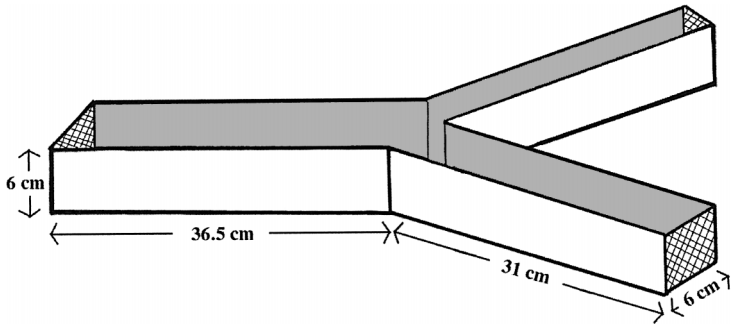


FIG. 1. Schematic diagram of the Plexiglas Y-maze used in behavioral assays to test responses of adult female *Eptesicus fuscus* to the odors of conspecifics collected from different colonies. Metal screens at each end of the Y-maze facilitated airflow through the maze.

trials, the maze was washed with ethyl alcohol to remove potentially confounding odors from previous trials.

Results of the behavioral assays were analyzed using a one-tailed binomial test (Zar, 1984; see Loughry and McCracken, 1991). When the difference in time spent in each arm of the maze was less than 30 sec, or the female did not move from the third arm of the maze, the choice was treated as a tie and excluded from subsequent analysis (Loughry and McCracken, 1991).

Each odor sample was analyzed using gas chromatography–olfactometry (GC-O) (Acree, 1997). With GC-O, gas chromatography was used to separate the components of a chemical mixture before being diluted in the humidified air of an olfactometer and blown into the face of a human detector or "sniffer." The sniffer assigned a descriptor to the perceived odor and recorded a chromatographic retention time. With the exception of a few diastereoisomers, the retention time is distinctive for each chemical. Because the odor of most chemicals is unique, when two chemicals share the same odor as well as the same retention time, they are assumed to be the same (Acree, 1997). This made it possible for us to compare the presence or absence of like chemicals across samples. GC-O involves little sample preparation time and is often more sensitive than standard methods of chemical detection such as gas chromatography–mass spectometry (GC-MS) (Pollien et al., 1999). Because a human sniffer was used, only chemicals that have odors sensed by humans were detected using GC-O. Thus, a large group of odorless chemicals was ignored, simplifying analysis and comparison across samples.

All samples were analyzed using a GC-O system designed by DATU, Inc. (Geneva, New York). One of us (J.M.B) served as the sniffer, so that descriptors assigned to each odor could be consistent across all samples. Each sample was analyzed by GC-O in duplicate, and only those responses that occurred in both runs

were used for making colony comparisons. Samples were coded so that colony affiliation was unknown at the time of analysis.

Samples were injected (splitless mode) on a 0.25-mm × 10-m OV-101 (HP1) column held at 35°C for 5 min and then programmed at 6°C/min to 225°C in a HP 5890 modified by DATU, Inc. The effluent was sniffed in a 5-liter/min air flow at approximately 75% relative humidity, and all responses were standardized in Kovats retention indices using C_7–C_{18} normal parafins (Acree, 1997).

The presence and character of each odor was recorded as the mean retention index for the response with a single descriptor. Fifteen odors were detected and measured for each sample. In addition, the duration of odor detection was recorded by the sniffer and used as a semiquantitative measure for each odor. The duration of time that a sniffer detects an odor corresponds to the range of retention indices (peak width) over which a odor is eluted. This peak width is roughly associated with the amount of the chemical that produced the odor in the sample. Factors that may have biased these measurements include: (1) overestimation of the most polar odor caused by peak broadening on methyl silicon, (2) differences in the odor thresholds of bats and humans, and (3) selective loss of volatiles in the sampling procedure. Nevertheless, using presence or absence, an odor description and a semiquantitative measure of the amount of odor were recorded for each of 15 chemicals.

Principle component analysis was first used to collapse the 15 odor variables, followed by discriminant function analysis of the first four principle components. Cross-validation was used to estimate error rates and to control for the potential bias associated with using the same set of observations to classify as well as to generate the discriminant function (Johnson and Wichern, 1992). Principle component analysis and discriminant function analysis were conducted using the SAS statistical package (SAS Institute Inc., 1990).

The average retention index and odor descriptor for each of the 15 chemicals analyzed in this study were then compared to published OV-101 retention indices and descriptors for known compounds found on the Flavornet database (Acree and Arn, 1997). When the retention index and descriptor were identical or similar, a possible odor match was indicated. A literature search for these chemicals was conducted to determine if there was published evidence that would suggest biological activity in other systems.

RESULTS

Female big brown bats (*Eptesicus fuscus*) selected one arm of the Y-maze (difference in time spent between arms >30 sec) during 127 trials using 117 different individuals. Individual females spent more time in the arm of the maze that contained the odor from their own colony-mate in a majority (77%; $N = 98$) of trials (one-tailed binomial test; $P < 0.001$).

Our banding records indicate that many adult females and female young-of-the-year return each summer to the same maternity roost. Of 108 adult females banded in 1996, 70 were recaptured as adults at the same colony in 1997 and, among these, 34 were recaptured in 1998. Twenty-two of the 182 female pups banded in 1996 returned to their natal colony as adults in 1997. Among these 22 females, 17 females showed signs of lactation. The following year, 10 of these females were recaptured and showed signs of lactation or postlactation. Fourteen of the 230 female pups banded in 1997 were recaptured as adults in 1998, and 10 were reproductively active. Only four pups were recaptured at a colony other than the colony of initial capture. Two were individuals who moved from the Milford A to Milford B roost. The other two were adult females banded at Harvard in 1996. One was captured at Milford A, and the other was captured in Milford B in 1997. Switching between roosts was extremely rare, and those recorded may represent errors in the recording of band numbers.

Because the extracts of swabs that contained odor samples were well below the sensitivity of GC-MS, we used human GC-O to detect and semiquantify these odors. The presence and quantity of 15 odors from 20 individual samples were used in principle component analysis. Chromatograms were generated for each sample and indicated the presence and quantity (duration of elution time/peak width) for odors shared among individuals. For example, one female from the Harvard colony and another from the Sterling colony shared seven common odors that differed in quantity (Figure 2). None of the four control swabs indicated a detectable odor when analyzed using GC-O. The first four principle components accounted for 67.9% of the variation and were subsequently used in discriminant function analysis (Table 1). Examination of character loadings for the first four principle components revealed that the first principle component was most heavily loaded with the peak widths of the chemicals eluting at retention times 848 and 1007. The second principle component was most heavily loaded with the chemical at retention time 1187 and most negatively associated with the odor emitted at 1302. Odors detected at 1076 and 1149 both loaded most strongly on the third principle component, and those detected at 772 and 1145 loaded most heavily on the fourth

TABLE 1. PRINCIPAL COMPONENT ANALYSIS OF VOLATILE ODORS IN *E. fuscus*[a]

Component	Eigenvalue	Difference	Proportion	Cumulative variation
Prin 1	3.21536	0.329315	0.214357	0.21436
Prin 2	2.88604	0.643554	0.192403	0.40676
Prin 3	2.24249	0.402898	0.149499	0.55626
Prin 4	1.83959	0.557045	0.122639	0.67890

[a] Measurements of 15 volatile odors from female *Eptesicus fuscus* were collapsed to four principle components. The first four principle components describe 67.9% of the variation and were used in discriminant function analysis.

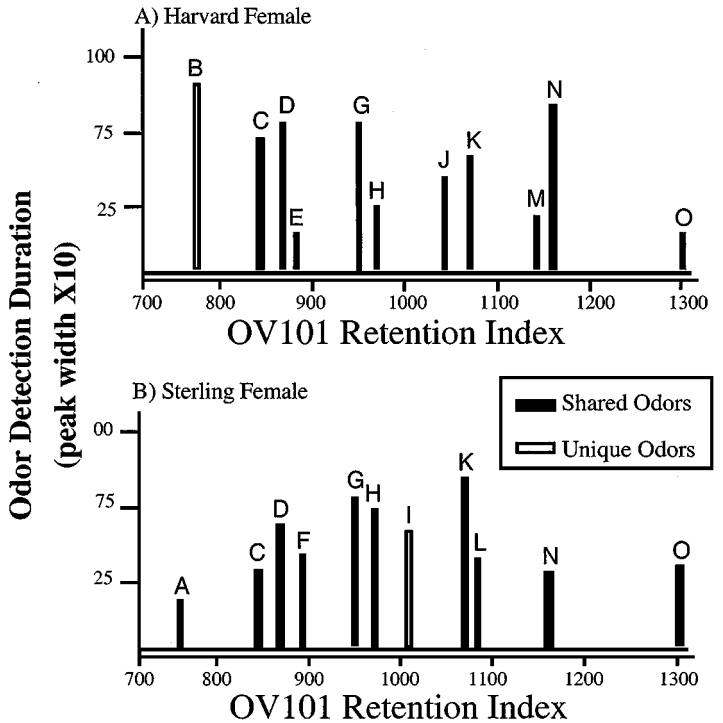


FIG. 2. GC-O chromatograms from two adult female *Eptesicus fuscus* collected from two different maternity colonies in central Massachusetts. These two females shared seven common chemicals (as determined by retention time and descriptions of odors) and quantity (peak width). Letters refer to variables listed in Table 2.

principle component (Table 2). While individuals varied in the relative contribution of each odor component (Figure 2), principle component analysis clearly revealed a separation of odor signature by colony (Figure 3).

Using the first four principle components, discriminant function analysis with cross-validation correctly classified 100% of the bats analyzed from Sherborn ($N = 6$), Harvard ($N = 4$), Milford A ($N = 3$), and Milford B ($N = 3$). For the Sterling colony ($N = 4$), two individuals were incorrectly classified as belonging to the Milford A colony.

From similarities in odor descriptors and OV-101 retention indices, likely chemical matches were found for 14 of the 15 odors used in the colony analysis. A literature search of these compounds shows that many of the chemicals identified as being potential matches were important in other biological systems (Table 3).

TABLE 2. RETENTION TIMES, DESCRIPTORS, AND PCA LOADINGS FOR THE 15 VARIABLES[a]

Variable	Retention time	Descriptor	Component loadings			
			Prin 1	Prin 2	Prin 3	Prin 4
A	772	Fruity	0.2136	−0.2632	0.0982	−0.4592
B	788	Sweet	−0.2365	−0.2106	−0.3235	0.0664
C	848	Musty	0.4586	0.2158	0.0577	−0.8761
D	870	Sweet/Plastic	0.2603	−0.1714	0.2758	−0.1721
E	884	Musty	0.2457	−0.1505	−0.0369	0.3529
F	896	Corn Meal	−0.2775	0.0870	0.1954	0.1029
G	949	Musty/Plastic	0.2096	0.0906	0.1698	0.2319
H	960	Eggplant	0.2690	0.2090	0.1629	0.3615
I	1007	Musty	0.4170	−0.0484	0.0900	0.2673
J	1049	Burning	−0.3587	0.2396	0.2781	0.2739
K	1076	Maple	0.0626	0.3243	−0.5374	−0.0478
L	1187	Fruity	−0.1191	−0.5144	−0.1803	0.1395
M	1145	Burning	0.0349	0.0759	0.2376	−0.4938
N	1149	Plastic	0.0507	0.3192	−0.5415	−0.0670
O	1302	Urine	0.2055	0.4692	0.2420	−0.1063

[a] The 15 variables were used in the principle component analysis of odors detected in female *Eptesicus fuscus*. The variables that load most heavily on the first four principle components are underlined. The first principle component is most heavily loaded for variables C and I; the second principle component for variables L and O; the third for K and N and the fourth for A and M.

DISCUSSION

It has become increasingly evident that olfactory cues are used by bats to identify individual colony members (Schmidt, 1988; Brooke and Decker, 1993), as territorial markers (Höller and Schmidt, 1993; Haffner, 1995; French and Lollar, 1998), and in mother–infant recognition (e.g., Watkins and Shump, 1981; Gustin and McCracken, 1987; Esser and Schmidt, 1989; De Fanis and Jones, 1995a; see Bloss, 1999 for a review). Olfactory cues may also be used by bats to complement auditory cues associated with mother–infant recognition (e.g., Thomson et al., 1985; Balcombe, 1990; Jones et al., 1991; Balcombe and McCracken, 1992; De Fanis and Jones, 1996).

This study provides the first evidence that female big brown bats, recognize the scent from members of their own colony. De Fanis and Jones (1995b) found similar results in the common pipistrelle, *Pipistrellus pipistrellus*. As in the study of De Fanis and Jones (1995b), we were not able to determine whether bats responded to the odor of familiar individuals or whether a group-distinct odor was recognized. Notwithstanding, the similarity among odors produced by members of the same colony of *Eptesicus fuscus* is supported by our chemical analysis. Discriminant function analysis was successful in grouping odor signatures by

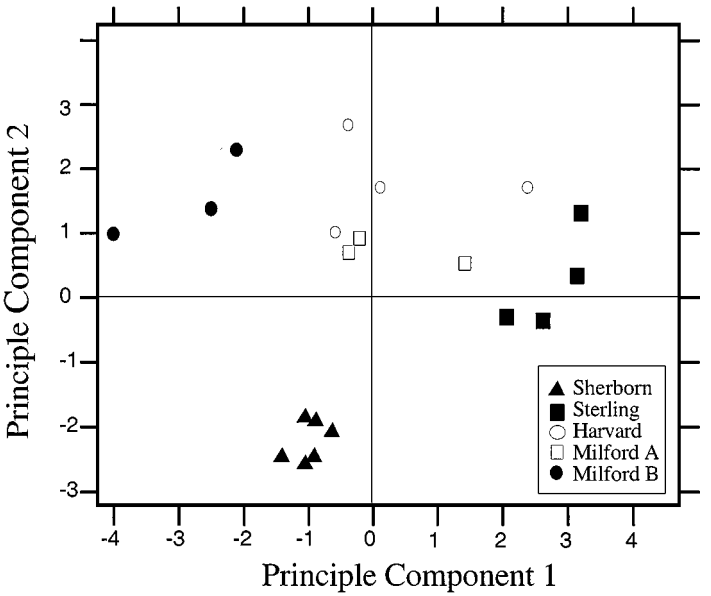


FIG. 3. Principle component analysis of the overall body odor female *Eptesicus fuscus* was used to collapse 15 measured odor variables into four components (see Table 1). This analysis reveals a separation of colonies by the first and second principle components.

the correct colony in a majority of cases, providing evidence for colony-specific odor signatures.

If colony members share similar odor signatures with one another more than they do with conspecifics from other colonies, the logical question becomes what factors produce these similarities? The consistency of odors among colony mates may be due to similarity in microbial cultures, shared food sources, identical roosting substrate, genetic relatedness, or a combination of any or all of the above.

Odors may be derived from several sources. Secretions from specialized glands, urine, or feces or by-products of microbial metabolism may all contribute to the overall odor of an individual. For example, glandular pockets of the fish-eating bat, *Noctilio leporinus*, contain pungent odors (Brooke and Decker, 1993, 1996). When bacteria from these pockets have been cultured, the odors produced were similar to those produced by the bats that were sampled (Studier and Lavoie, 1984). Thus, group-specific odors may reflect unique bacterial cultures shared by grooming or roosting in proximity to members of the same colony (Dapson et al., 1977; Studier and Lavoie, 1984). Members of the genus *Eptesicus* possess well-developed facial and pararhinal glands (Quay, 1970), which may contribute to odor production.

TABLE 3. POSSIBLE CHEMICALS ASSOCIATED WITH ODORS DETECTED FROM GC-O ANALYSIS OF *Eptesicus fuscus*[a]

RI	Possible compound(s)	Biological activity in other systems
772	Methyl-2-methylbutanoate	?
788	Ethyl butanoate	Dung beetle attractant (Burger et al., 1995)
848	2-Methyl-1-butanol	Sap beetle attractant (Nout and Bartelt, 1998; Bartelt and Wicklow, 1999)
870	(*E*)-2-Hexene-1-ol	Green capsid bug attractant (Groot et al., 1999); spined soldier bug attractant (Sant'Ana et al., 1999)
884	Ethyl valerate	?
896	[A]2-Furfurylthiol or [B]2-acetyl-1-pyrroline	[A]Corn volatile (Buttery and Ling, 1998); [B]mousy aroma in wines (Herderich et al., 1995); maize volatile (Bredie et al., 1998)
949	Heptanol	?
960	[A]6-Methyl-5-hepten-2-one or [B]1-octen-3-ol	[A]Volatile involved in attracting aphid-tending ants (Cordova-Yamauchi et al., 1998); ant pheromone (Do Nascimento et al., 1998); aphid parasitoid attractant (Du et al., 1998); aphid spacing chemical (Gonzales et al., 1999); [B]vertebrate volatile attractant to mosquitos (Takken, 1999) and ticks (Osterkamp et al., 1999); green capsid bug attractant (Groot et al., 1999)
1007	Phenylacetaldehyde	Sternal gland secretion in male koalas (Salamon and Davies, 1998); fall armyworm attractant (Meagher and Mitchell, 1998); floral scent attracting Lepidoptera (Omura et al., 1999a,b) and Hymenoptera (Meagher and Mitchell, 1999)
1049	(*E*)-Ocimene	Volatile released by damaged cotton plant (Rose et al., 1998) or broad bean (Du et al., 1998) which attracts herbivore enemies
1076	2-Phenylethanol	Sheep blowfly attractant to host odor (Park and Cork, 1999); pineapple beetle (Zilkowski et al., 1999), house fly (Chapman et al., 1998), twelve-spotted lady beetle and green lacewing attractant (Zhu et al., 1999)
1087	Linalool	Volatile released by damaged plants is an attractant for spined soldier bugs (Sant'Ana et al., 1999); lavender compound used in aromatherapy (Lis-Balchin and Hart, 1999); green capsid bug attractant (Groot et al., 1999); volatile used by Egyptian cotton leaf worm to decriminate between damaged and undamaged plants (Jonsson and Anderson, 1999); moth-pollinated flower volatile attractant to hawkmoths (Raguso et al., 1996; Raguso and Light, 1998)
1145	Benzyl acetate	Moth-pollinated flower volatile attractant to hawkmoths (Raguso et al., 1996; Raguso and Light, 1998)
1149	?	?
1292	*O*-Aminoacetophenone	Component of Japanese weasel anal sac (Acree et al., 1990); mountain beaver (Nolte et al., 1993) and bird repellent (Clark, 1998)

[a] The potential compounds were determined using the descriptors and OV-101 retention times listed on the Flavornet (Acree and Arn, 1997). Many of these chemicals have been shown to be biologically active odorants in other systems. The retention index (RI) refers to the average RI used in this study and corresponds to the retention times listed in Table 2. A question mark denotes that the RI and odor desciptors did not match any listed on the Flavornet or the compound was not found in recent literature.

By a similar mechanism, members of a colony that forage in the same general area may share a similar diet. Diets of different big brown bat colonies are known to differ in insect composition (Whitaker, 1995). The metabolism of related food items, especially by related individuals could produce similar volatile by-products and, thus, affect overall body odor (Schellinck et al., 1997). In our study, two members of the Sterling colony were misclassified into the Milford A colony. These colonies are separated by approximately 45 km. Individuals from the two Milford colonies, separated by only 2 km were not misidentified as belonging to the other Milford maternity roost. This evidence suggests that shared diet may not be an important factor in determining colony odor. However, further investigations using dietary controls are needed to assess this possible source of odor production, since even bats from the same roost may forage in different locations and consume varied diets. In addition, roosting substrate should be evaluated, as individuals in our study may have responded to the familiar odor of their roost in behavioral tests.

Female *Eptesicus fuscus*, especially those from maternity colonies in human-made structures, tend to show high site fidelity during pregnancy and lactation (Brigham and Fenton, 1986). After disturbance, colony members often relocate as a group, indicating that roosting assemblages may function as social groups rather than random aggregations of individuals (Brigham and Fenton, 1986). Since our banding records support female philopatry, inhabitants of these maternity roosts are likely to be genetically related. Colony-specific scents could be the result of genetically related individuals that produce similar odors.

The major histocompatibility complex (MHC) has been shown to affect the odor of an individual (Eggert et al., 1996, 1998). MHC is a highly polymorphic group of genes that controls the immune system's recognition of self and nonself (Eggert et al., 1998; Penn and Potts, 1999). Although the precise mechanism for how MHC loci influence odor production is not entirely clear, based on olfactory cues, some rodents and humans are able to distinguish individuals who vary in this gene complex (Eggert et al., 1998; Penn and Potts, 1998). A similar mechanism might exist in bats if chemosensory recognition of kin occurs among individuals that share similar MHC regions. Further studies are needed to elucidate such a recognition system in bats, and a first step would be to evaluate the degree of genetic relatedness among colony members.

Group recognition via olfactory or other cues could facilitate the stability of social groups. Familiar individuals are more likely to cooperate and avoid agonistic interactions than those with infrequent contact or that are unrecognized (Lewis, 1995). Roosting groups are important for bats, especially in maternity colonies where stable temperatures are vital for proper development of the young (Kunz and Hood, 2000). The cost of thermoregulation is decreased by clustering behavior (Herreid, 1967; Trune and Slobodchikoff, 1976; Bonaccorso et al., 1992; Lewis, 1995). The recognition of colony mates may also facilitate winter aggregations in hibernacula.

When the possible chemical identities of the 15 odors used in the study were investigated, 11 of the 14 potentially identified compounds were found to be biologically active in other taxa (Table 3). For example, the odor described as musty, with an average retention index of 1007 (Table 2), is probably phenylacetaldehyde with a retention index of 1004 (Acree and Arn, 1997). This compound was identified as a heavily loaded variable in the first principle component (Table 2) and may be important in colony recognition. Phenylacetaldehyde is present in the secretion from the sternal gland of male koalas (Salamon and Davies, 1998). It is also an attractant for many lepidopteran (Omura et al., 1999a,b) and hymenopteran species (Meagher and Mitchell, 1999). The biological importance of an odor among diverse taxa is not surprizing given the homology that exists in the olfactory detection systems of a wide diversity of organisms (Acree and Bloss, 1996).

Most of what is understood about pheromones comes from the study of insects. This group accounts for about 70% of all described species, and many of these are agricultural crop pests (Agosta, 1992). Since considerable research is devoted to understanding insect attractants and repellents, most compounds identified as potential chemicals in big brown bat colony odor also were considered to be important in insect systems (Table 3). In fact, many of the insect families represented in Table 3 have been reported in the diet of *E. fuscus* (Whitaker, 1995; W. R. Hood, unpublished data). As the study of chemical ecology expands, many insect pheromones have been shown to be biologically important in vertebrates (Acree and Bloss, 1996).

Our study does not unequivocally demonstrate that colony members recognize each other using olfactory cues or that group-distinct colony odors are present. However, it does suggest that female big brown bats prefer the odor of a roost-mate to that of a conspecific from another colony. Such a preference may promote colony stability and, thus, have implications for foraging, kin recognition, and mating. Olfactory cues, like the well-studied auditory cues of bats may be important in establishing and maintaining social interactions within a species.

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MIMICRY OF HOST CUTICULAR HYDROCARBONS BY SALTICID SPIDER *Cosmophasis bitaeniata* THAT PREYS ON LARVAE OF TREE ANTS *Oecophylla smaragdina*

RACHEL A. ALLAN,[1] ROBERT J. CAPON,[2] W. VANCE BROWN,[3] and MARK A. ELGAR[1,*]

[1]*Department of Zoology*
[2]*Department of Chemistry*
University of Melbourne
Parkville, Victoria 3052, Australia
[3]*CSIRO Entomology*
GPO Box 1700, Canberra, ACT 2601, Australia

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Abstract—The salticid spider *Cosmophasis bitaeniata* preys on the larvae of the green tree ant *Oecophylla smaragdina*. Gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS) reveal that the cuticle of *C. bitaeniata* mimics the mono- and dimethylalkanes of the cuticle of its prey. Recognition bioassays with extracts of the cuticular hydrocarbons of ants and spiders revealed that foraging major workers did not respond aggressively to the extracts of the spiders or conspecific nestmates, but reacted aggressively to conspecific nonnestmates. Typically, the ants either failed to react (as with control treatments with no extracts) or they reacted nonaggressively as with conspecific nestmates. These data indicate that the qualitative chemical mimicry of ants by C. bitaeniata allows the spiders to avoid detection by major workers of O. smaragdina.

Key Words—Chemical mimicry, cuticular hydrocarbons, nestmate recognition, inter-specific exploitation, predation.

INTRODUCTION

The resident workers of many species of ants attack and kill nonnestmates that try to enter the nest (e.g., Jaisson, 1991). Nevertheless, there are many records of nonnestmates living within the nests of these species (see Hölldobler and Wilson, 1990 for review). Nonnestmates survive inside these nests if they can

*To whom correspondence should be addressed. E-mail: m.elgar@unimelb.edu.au

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prevent the resident ants from attacking them or can defend themselves against ant attack (Eisner et al., 1978). This can be achieved through a combination of morphological and chemical mimicry, defensive chemicals, appeasement secretions, and behavioral strategies (reviews in Kistner, 1979; Dettner and Liepert, 1994; Haynes and Yeargan, 1999; Lenoir et al., 2001).

Cuticular hydrocarbon mimicry is the most commonly recognized mechanism that myrmecophiles and termitophiles use to deceive their social insect hosts (Dettner and Liepert, 1994). Resident workers routinely attack conspecifics and other species whose cuticular hydrocarbons are qualitatively and/or quantitatively different from those of the nest inhabitants (Henderson et al., 1990; Bagneres et al., 1991; Bonavita-Cougourdan et al., 1993, 1997; Habersetzer, 1993; Takahashi and Gassa, 1995; Vauchot et al., 1996). Thus, access to the nest by myrmecophiles and termitophiles requires a degree of mimicry of the cuticular hydrocarbons of the host (e.g., Hölldobler, 1971).

The cuticular hydrocarbon profiles of several ants and termites and their associates have been identified by gas chromatography–mass spectrometry (GC-MS), but these studies did not include recognition bioassays (e.g., Howard et al., 1980, 1990a,b; Vander Meer et al., 1989; Howard, 1992; Howard and Infante, 1996; Bagneres et al., 1996; Völkl et al., 1996; Vauchot et al., 1998; Lenoir et al., 2001). Simply revealing similarities between species in their cuticular hydrocarbons is not sufficient to demonstrate that the cuticular hydrocarbons function as camouflage or that they represent species or colony-specific recognition cues. Cuticular hydrocarbons may be acquired by a myrmecophile simply as a by-product of living in an ant colony and may not communicate any signal to the ant. Thus, recognition bioassays are essential to demonstrate that chemical mimicry or camouflage occurs when the cuticular hydrocarbons of a myrmecophile resemble those of its host. Other studies that have examined morphological and/or behavioral traits involved in the deception of ants by myrmecophiles have generally assumed that chemical mimicry or chemical camouflage is also involved (Garnett et al., 1985; Breed et al., 1992; Völkl and Mackauer, 1993; Feener, 1995; Völkl, 1995; Weissflog et al., 1995).

More recent studies have combined chemical analyses with behavioral bioassays, and they reveal that two different groups of chemical compounds may be used in nestmate recognition. Moritz et al. (1991) show that the death's-head hawk moth, *Acherontia atroposa*, which parasitizes colonies of the honeybee, *Apis mellifera*, chemically camouflages itself in a bouquet of fatty acids that makes the moth chemically "invisible" to the bees. However, it was not determined whether the bees used these fatty acids in nestmate recognition. Liepert and Dettner (1993) demonstrated with recognition bioassays that the aphidiid wasp *Lysiphlebus cardui* uses chemicals located on its cuticular surface to avoid being attacked by the ant *Lasius niger.* This ant guards the aphids that the wasp parasitizes. The chemicals were subsequently identified as methyl-branched alkanes rather than fatty acids

(see Liepert and Dettner, 1996). These studies indicate that nestmate recognition and the deception of social insects is mediated by both methyl-branched alkanes and fatty acids (see also Bonavita-Cougourdan et al., 1987; Franks et al., 1990).

It is generally thought that host cuticular hydrocarbons are acquired by the myrmecophile during contact with the host (Vander Meer et al., 1989; Akino et al., 1996). Most myrmecophiles come into direct physical contact with their host (Vander Meer and Wojcik, 1982; Vander Meer et al., 1989; Franks et al., 1990; Howard et al., 1990a,b), and the host often attacks myrmecophiles before they have acquired the mimetic cuticular hydrocarbons (Franks et al., 1990). These ant-derived hydrocarbons are probably not cuticular but rather originate from the hydrocarbon-rich postpharyngeal glands of the attacking ants (Howard et al., 1990b). The postpharyngeal hydrocarbons are similar to the cuticular hydrocarbons. Indeed, if the contents of the postpharyngeal gland are spread onto the cuticle of an ant, it will be accepted as a nestmate (Soroker et al., 1994; Hefetz et al., 1996).

The importance of chemical cues in the deception of ants by a brood predator that avoids direct contact with its host remains poorly understood (Akino et al., 1999; Elmes et al., 1999). The salticid spider *Cosmophasis bitaeniata* lives within the nests of the ant *Oecophylla smaragdina*, where it preys on the ant larvae but avoids direct contact with the workers (Allan and Elgar, 2001). Workers of *O. smaragdina* construct nests by binding together leaves with silk produced by larvae; a mature colony of *Oecophylla* may contain between 100,000 and 500,000 workers (Hölldobler and Wilson, 1978) that may construct as many as 150 nests across 12 trees (Way, 1954). Major workers of *Oecophylla* aggressively defend their colony from intruders of other species or conspecifics from neighbouring colonies (Hölldobler, 1983; Dejean, 1990; Keegans et al., 1991). Major workers attack most arthropods they encounter and consequently reduce the numbers of insects on the trees they inhabit (Lokkers, 1986, 1990). Individuals of *C. bitaeniata* are able to enter nests of ants apparently undetected. Here, we investigate whether the cuticular chemicals (hydrocarbons or fatty acids) of *C. bitaeniata* mimic those of *O. smaragdina* by using gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS). Recognition bioassays are conducted to examine whether the relationship between *C. bitaeniata* and *O. smaragdina* is facilitated by chemical mimicry and whether *C. bitaeniata* is able to mimic the nestmate recognition cues of its host.

METHODS AND MATERIALS

Gas Chromatography. The cuticular chemicals of the spider *C. bitaeniata* and the major workers, minor workers, and larvae of *O. smaragdina* were extracted with hexane and then analyzed by using capillary gas chromatography. Individuals of each species and caste were collected from the campus of James Cook University in Townsville, Australia. Immediately after collection, the ants, larvae, and spiders

were immersed in separate vials, each containing 100 μl of HPLC-grade hexane for 10 min, then each vial was agitated for 1 min on a vortex mixer before the specimens were removed. The hexane solution was evaporated under a stream of nitrogen. Immediately prior to analysis, 10 μl of hexane was added to each sample and 1 μl of this solution analyzed by gas chromatography using a Shimadzu 17A gas chromatograph with a 5% phenyl and 95% methyl siloxane stationary phase (S.G.E. BPX5, 25 m $\times$ 0.33 mm ID; 0.5 μm). The temperature program ramped from 60°C to 300°C at 20°C/min. The temperature was held constant for 3 min at 300°C prior to ramping at a rate of 2°/min to 330°C where it was held constant for 10 min. The retention time was 40 min. The injector was operated in splitless mode for 1 min at 350°C. The detector was kept at 375°C.

Gas Chromatography–Mass Spectrometry (GC-MS). Mass spectra were determined on a VG Micromass 70-70F mass spectrometer interfaced directly to a Hewlett-Packard 5792A capillary gas chromatograph and a VG 11-250 data system. The column used was a bonded-phase capillary methylsilicone (Alltech Econocap, SE30; 25 m $\times$ 0.33 mm ID; 0.25 μm). Injections of the hexane solutions were made on-column at 60°C using a Hewlett-Packard 19290A on-column inlet. Electron ionization mass spectra were obtained at an ionization voltage of 70 eV and a source temperature of 200°C. The mass spectra in conjunction with ECL values were used to determine the chemical identity of the individual hydrocarbons.

Behavioral Bioassay. The bioassays examined the response of foraging *O. smaragdina* major workers to discs of filter paper, thereby ensuring that chemical cues only were detected. The cuticular hydrocarbons were extracted from the animals using hexane, and 1-cm discs of filter paper were then submerged in the supernatant. There were four treatments comprising extracts from: (1) 15 nestmate major workers collected from one colony of *O. smaragdina*, (2) 15 nonnestmate major workers collected from another colony of *O. smaragdina*, (3) 15 *C. bitaeniata* collected from the same colony as the nestmate major workers, and (4) control filter papers immersed in hexane alone. There were insufficient numbers of spiders available to examine intercolony variation, and, thus, each ant and spider pair was collected from a different nest, but all of the nests sampled within each treatment were collected from a single colony.

Field-collected ants and spiders were cooled at 4°C for 5 min prior to cuticular hydrocarbon extraction. All 15 animals from each treatment were immersed in 2 ml of hexane for 10 min, followed by 1 min of agitation on a vortex mixer. The animals in each treatment had to be pooled to ensure that the concentration of cuticular hydrocarbons on the individual filter papers would be equal to that of a single worker, since losses can occur with solvent extraction procedures (Brill and Bertsch, 1985). This procedure resulted in one sample for each treatment. The total extractable cuticular hydrocarbon content per animal was estimated by comparing GC response factors for animal extracts with known concentrations

of standard hydrocarbons (SGE standards (up to C_{30}) supplied by Alltech). This analysis indicated that individual ants and spiders possessed a total extractable cuticular hydrocarbon content of ~400 ng/spider and ~750 ng/major worker.

The hexane extract from each treatment was separated through a column of silica gel (Biosil) to remove polar and glandular constituents that may influence the responses of foraging workers. These four samples were then concentrated to 20 μl under a stream of nitrogen. The resulting 20 μl was pipetted in equal 2-μl aliquots onto ten, 1.27-cm-diam. discs of Whatman filter paper. The filter paper discs were left at room temperature for 10 min to ensure that the hexane evaporated. Individual filter papers were placed onto the foraging trails of major workers using forceps. Ground foraging trails were chosen in preference to tree foraging trails to prevent the discs of filter paper falling from trees. The number of foraging major workers of *O. smaragdina* that walked within 1 cm of the disc of filter paper was vocally recorded onto a dictaphone for 3 min. The immediate response of these foraging ants to the disc of filter paper was also recorded.

RESULTS

Cuticular Hydrocarbons. The cuticular hydrocarbon profiles of the major and minor workers, larvae of *O. smaragdina* and *C. bitaeniata* are shown in Figure 1. These profiles show little similarity up to a retention time of about 20 min, and since the components represented are present in ants (particularly the workers), they are likely to be derived from gland material. These early components are predominantly shorter chain *n*-alkanes, and such lipids are well known from the Dufour glands. In contrast, 14 peaks with retention times of 20–40 min and ECLs of 2700 to 3755 were routinely present in every chromatogram. The spider *C. bitaeniata* did not contain any species-specific cuticular hydrocarbons, and its components were identical with those numbered peaks of *O. smaragdina*.

The structures of the components represented by these 14 peaks were identified by GC-MS (Table 1) and are all well-known components of insect cuticular hydrocarbons (see Lockey, 1988). Only three hydrocarbon types were present; peak 1 represented the *n*-alkane heptacosane, and the other 13 were all either internally branched monomethylalkanes or internally branched dimethylalkanes with chain lengths from C_{29} to C_{37}. Branching showed the usual pattern of being exclusively on odd-numbered carbon atoms when the chain contained an odd number of carbons, and on both odd and even numbered carbon atoms when the chain length was even. All the dimethylalkanes showed a three methylene unit separation between the two branching positions. In all cases these peaks represented mixtures of isomers. However, for convenience we simply refer to them as single components.

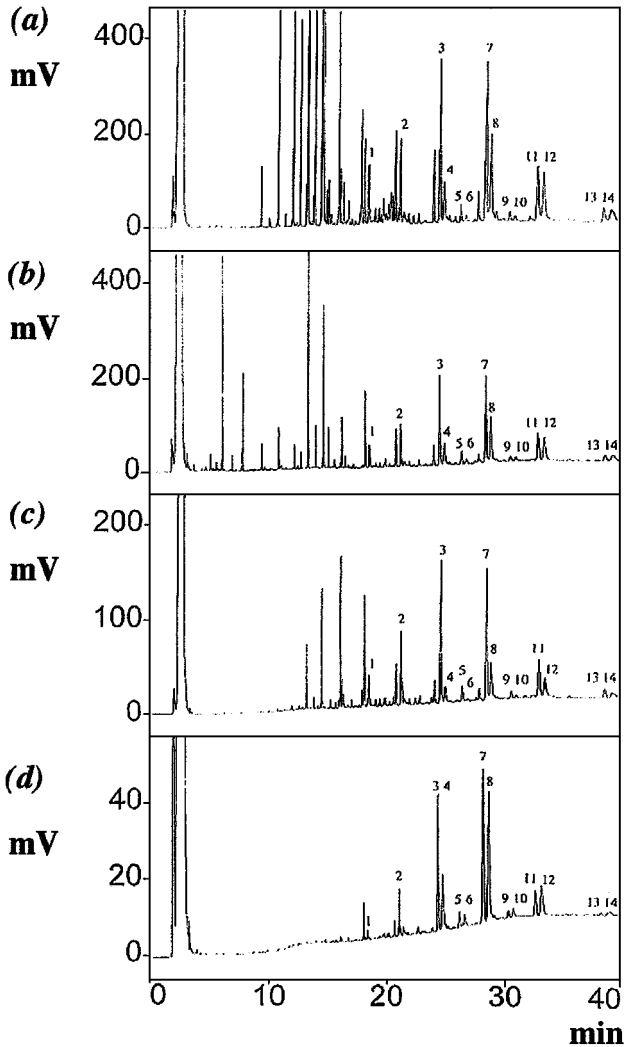


FIG. 1. Gas chromatogram of (a) major workers of *O. smaragdina*, (b) minor workers of *O. smaragdina*, (c) larvae of *O. smaragdina*, and (d) *C. bitaeniata*. Each peak in (d) is marked from 1 to 14, and peaks in common with *C. bitaeniata* are labeled in the other traces.

Behavioral Bioassay. The number of foraging workers of *O. smaragdina* that came within 1 cm of the filter papers was not significantly different among treatments (Table 2). The extracted hydrocarbons on the filter papers would have low vapor pressure, thus producing a small active space and be detected only by

TABLE 1. HYDROCARBON CONSTITUENTS IDENTIFIED BY GC-MS OF 14 PEAKS USED IN COMPARISONS BETWEEN *C. bitaeniata* AND *O. smaragdina*

Peak[a]	Hydrocarbon	ECL[b]	Diagnostic ions[c]
1	*n*-C_{27}	27.00	380
2	15-; 13-; 11-; 9(t)-; 7-MeC_{29}	29.32	224/5(s); 196/7, 252/3; 168/9, 280/1; 140/1, 308/9; 112/3, 336/7
3	15-; 13-; 11-; 9(t)-; 7-MeC_{31}	31.35	224/5, 252/3; 196/7, 280/1; 168/9, 308/9; 140/1, 336/7; 112/3, 364/5
4	13-17-; 11,15-diMeC_{31}	31.63	196/7, 224/5, 266/7, 294/5; 168/9, 252/3, 323
5	16-; 15-; 14-; 13-; 12-; 11-; 10-MeC_{32}	32.31	238/9, 252/3; 224/5, 266/7; 210/1; 196/7, 294/5; 182/3, 308/9; 168/9, 322/3; 154/5, 336/7
6	13,17-; 12,16-; 11,15-diMeC_{32}	32.57	196/7, 238/9, 267, 309; 182/3, 252/3(s), 323; 168/9, 238/9, 267, 337
7	17-; 15-; 13-; 11-; 9-; 7-MeC_{33}	33.35	252/3(s); 224/5, 280/1; 196/7, 308/9; 168/9, 336/7; 140/1, 364/5; 112/3, 392/3
8	15,19-; 13,17-; 11,15-diMeC_{33}	33.64	224/5, 294/5(s); 196/7, 252/3, 266/7, 323; 168/9, 238/9, 280/1, 351
9	17-; 16-; 15-; 14-; 13-; 12-; 11-MeC_{34}	34.30	252/3, 266/7; 238/9, 281; 224/5, 294/5; 210/1, 308/9; 196/7, 322/3; 182/3, 336/7; 168/9, 350/1
10	15,19-; 14,18-; 13,17-; 12,16-; 11,15-diMeC_{34}	34.56	224/5, 238/9, 294/5, 309; 210/2, 252/3, 281, 323; 196/7, 266/7(s), 337; 182/3, 252/3, 281, 351; 168/9, 238/9, 294/5, 365
11	17-; 15-; 13-; 11-MeC_{35}	35.34	252/3, 280/1; 224/5, 308/9; 196/7, 336/7; 168/9, 364/5
12	15,19-; 13,17-; 11,15-diMeC_{35}	35.59	224/5, 252/3, 295, 323; 196/7, 266/7, 280/1, 351; 168/9, 238/9, 308/9, 379
13	17-; 15-; 13-; 11-MeC_{37}	37.30	252/3, 308/9; 224/5, 336/7; 196/7, 364/5; 168/9, 392/3
14	17,21-; 15,19-; 13,17-; 11,15-diMeC_{37}	37.55	252/3, 323(s); 224/5, 294/5, 281, 351; 196/7, 266/7, 308/9, 378/9; 168/9, 238/9, 336/7, 407

[a] Peak numbers correspond with those in Figure 1.
[b] Equivalent chain length.
[c] Diagnostic ions upon which structural assignments were made. (t) Indicates that only a trace amount of this component was present, and (s) indicates symmetrical fragment ions.

TABLE 2. RESPONSE OF FORAGING WORKERS OF *O. smaragdina* TO FILTER PAPER DISCS SOAKED IN EXTRACTED CUTICULAR HYDROCARBONS OF NESTMATES, NONNESTMATES, OR *C. bitaeniata*[a]

Behavior	Control[b]	*C. bitaeniata*	*O. smaragdina*		$F_{3,36}$
			Nestmates	Nonnestmates	
Number of visits by ants to the paper disc	14.2 ± 1.8	15.1 ± 1.6	11.1 ± 1.2	13.9 ± 2.3	0.95
Number of times visiting ants made no response	5.3 ± 1.1	5.1 ± 0.8	0.9 ± 0.3	0.7 ± 0.3	12.93**
Number of times visiting ants behaved nonaggressively	0.5 ± 0.3	1.2 ± 0.5	2.7 ± 1.0	0.7 ± 0.3	2.78*
Number of times visiting ants behaved aggressively	8.9 ± 1.0	6.3 ± 1.6	5.3 ± 1.7	17.1 ± 2.3	9.55**

[a] Values are means ± SE; each treatment had 10 replicates.
[b] Control discs were immersed in hexane only. *$P < 0.05$; **$P < 0.001$.

contact or at close range (Blum, 1987). We identified four categories of responses of the foraging major workers of *O. smaragdina*. Workers that simply walked within 1 cm of the disc of filter paper but did not stop or otherwise make any obvious response were classified as making no response to the disc. Workers that stopped and touched the disc of filter paper with their antennae but did not open their mandibles made a nonaggressive response. Workers that raised their abdomen, opened their mandibles, and/or bit the disc of filter paper were deemed aggressive, and we scored an aggression index by summing the number of each of these interactions.

The response of workers towards the filter papers depended upon the treatment (Table 2). Control discs and discs with the extracted cuticular chemicals of *C. bitaeniata* were ignored by foraging workers more frequently than discs with nestmate or nonnestmate treatments (Dunnett post-hoc comparison with nestmate treatment: *C. bitaeniata*, $q = 4.2$, $P < 0.001$; control, $q = 4.4$, $P < 0.001$; nonnestmate ant, $q = 0.2$, $P > 0.9$). In contrast, workers exhibited higher frequencies of nonaggressive interactions toward discs with the extracted cuticular chemicals of nestmates and were significantly more aggressive toward discs with the extracted cuticular chemicals of nonnestmates (Dunnett post-hoc comparison between nestmate and nonnestmate treatment: $q = 11.8$, $P < 0.001$).

DISCUSSION

The cuticular hydrocarbons of *C. bitaeniata* closely resemble those of its host ant *O. smaragdina*, and the ants react less aggressively to extracted cuticular hydrocarbons of nestmate workers and spiders than to nonnestmate workers. Further, workers of *O. smaragdina* become more active in the presence of the congenor spiders *C. micans* and *C. micarioides*, which do not associate with these ants, than when in the presence of either their nestmates or *C. bitaeniata* (Allan and Elgar, 2001). Since *C. bitaeniata* preys on the larvae of their host ant (Allan and Elgar, 2001), these data suggest that this myrmecophile spider is an aggressive chemical mimic. This is the first demonstrated example of this kind of mimicry by myrmecophile spiders (see Elgar, 1993; Cushing, 1997; see also Allan et al., 1996).

Fourteen hydrocarbons typically present in the gas chromatogram of *C. bitaeniata* were identified as *n*-alkanes and internally branched methylalkanes and dimethylalkanes. Despite the lack of volatility of hydrocarbons of this type, they are thought to be important in nestmate recognition for social insects (Bonavita-Cougourdan et al., 1987, 1993; Provost et al., 1994). While the 14 cuticular hydrocarbons of the spiders were present on the cuticles of the major and minor workers and the larvae of the host ant *O. smaragdina*, the latter shows some quantitative differences among castes. It is, therefore, possible that the mimicry of these ants by *C. bitaeniata* may be caste specific.

Myrmecophiles may have species-specific cuticular chemicals in addition to their host-resembling chemicals. For example, the lipids on the cuticle of the myrmecophilic cricket *Myrmecophilus* sp. comprise hydrocarbons and wax esters, but only the hydrocarbons decrease when the cricket is isolated from ants, suggesting that while the esters are specific to the cricket, the hydrocarbons may be acquired from the ants (Akino et al., 1996, see also Akino et al., 1999). In contrast, *C. bitaeniata* has no species-specific cuticular chemicals. Why some species that live with social insects possess species-specific cuticular chemicals and others do not (e.g., Vander Meer et al., 1989; Moritz et al., 1991) is not clear. However, there is some suggestion that termitophiles and myrmecophiles that biosynthesize their mimetic chemicals are less likely to have species-specific chemicals (Howard et al., 1990a).

The behavioral bioassay using discs of filter paper indicates that the chemical mimicry reduces the level of aggressive behavior directed towards the myrmecophiles by their host. Foraging major workers attacked the extracted cuticular hydrocarbons of nonnestmates but were not aggressive towards the extracted cuticular chemicals of nestmates, myrmecophiles, or controls. These data are consistent with those from other recognition bioassays that examined whether cuticular hydrocarbons are used by ants to discriminate between conspecific workers (Morel et al.,

1988; Takahashi and Gassa, 1995; Bonavita-Cougourdan et al., 1997; Thomas et al., 1999). However, the hexane extracts of *C. bitaeniata* are not perceived to be chemically identical to those of nestmate or nonnestmate workers. While foraging *O. smaragdina* workers more frequently failed to respond to the control and *C. bitaeniata* treatments than to the cuticular hydrocarbons of conspecifics, nonaggressive interactions were more frequently observed in the spider and nestmate treatments than in the control and nonnestmate treatments.

There are several possible explanations for these patterns. First, the foraging major workers may have responded to the spiders as if they were nonalarmed nestmate workers, but responded to the nestmates and nonnestmate ants as if they were alarmed. This might arise if the ants were able to release alarm pheromone shortly before being immersed in hexane. Second, the cuticular hydrocarbons of spiders may resemble a generalized species odor that does not contain a colony identification cue. Finally, the cuticular hydrocarbons of ants function as colony-recognition cues, although the spiders do not mimic the major worker caste, but rather the larvae or minor caste of *O. smaragdina*.

Resolving these alternatives depends in part on whether cuticular hydrocarbons alone act as colony-specific recognition cues in ants. The hexane extract of *C. bitaeniata* was filtered through silica gel and, thus, only contained cuticular hydrocarbons. Any behavioral response by foraging ants towards filter papers covered in spider extracts can, therefore, be attributed only to these components. However, the lack of response by the workers to these compounds suggests that hydrocarbons alone may not be used to distinguish between nestmates and nonnestmates. A distinction is often drawn between a species odor and a colony odor (e.g., Vander Meer and Wojcik, 1982; Habersetzer, 1993), and fatty acids and esters may be more important in recognition between colonies than cuticular hydrocarbons (Franks et al., 1990; Kaib et al., 1993). If spiders mimic *O. smaragdina* at the species level but not at the colony level, they could disperse to adjacent colonies without eliciting aggression from workers. Thus, the alkanes and internally branched methylalkanes and dimethylalkanes of *C. bitaeniata* may not be a colony-specific cue, but rather a more generalized species identification cue. However, this raises the question of which chemical cues are used by *O. smaragdina* to distinguish between nestmates and nonnestmate conspecifics.

It is possible that the cuticular chemicals of spiders did not elicit the same response from foraging workers as that of nestmates because *C. bitaeniata* does not mimic the major caste of *O. smaragdina.* Several myrmecophiles are chemical mimics of the larvae of their hosts (Hölldobler, 1971; Garnett et al., 1985; Vander Meer et al., 1989; Howard et al., 1990b). If the relative proportions of hydrocarbons differ between different castes within a species (Howard et al., 1982; Bonavita-Cougourdan et al., 1993; Haverty et al., 1996), then *C. bitaeniata* may mimic the relative proportions of the cuticular hydrocarbons of either the minor workers or the larvae of *O. smaragdina*.

Our data raise more general issues about the extraction of cuticular compounds for recognition bioassays involving ants and termites. Typically, these compounds are extracted using hexane or pentane (e.g. Thomas et al., 1999), and, thus, are at risk of being contaminated by glandular compounds. For example, the gas chromatograms of *O. smaragdina* have many compounds that are more volatile than the numbered cuticular compounds, all of which are missing from the chromatogram of *C. bitaeniata*. One procedure is to place the solvent extract through silica gel to remove nonhydrocarbon compounds, but this does not remove volatile hydrocarbons originating from glands (see Bonavita-Courgourdan, 1989; Bagneres et al., 1991). Evaporating the solvent (hexane or pentane) from extracts with nitrogen may eliminate volatile glandular hydrocarbons. However, volatile mandiblular and Dufour gland secretions may play a role in colony odor (Jaffe, 1983; Su and Haverty, 1991; Dani et al., 1996). Although glandular chemicals are generally not considered appropriate as a colony-specific cue due to their volatility (Smith and Breed, 1995), their presence in the separated hexane extract of the workers of *O. smaragdina* may have altered the behavior of the foraging major workers. Thus, recognition bioassays of cuticular hydrocarbons that rely on the extraction of chemicals in organic solvents (e.g., hexane) may be confounded by the presence or absence of noncuticular chemicals that may or may not play a role in colony and/or species recognition.

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CHARACTERIZATION OF (1*R*,4*S*,4a*R*,7*S*,7a*R*)-DIHYDRONEPETALACTOL AS A SEMIOCHEMICAL FOR LACEWINGS, INCLUDING *Chrysopa* spp. AND *Peyerimhoffina gracilis*

A. M. HOOPER,[1] B. DONATO,[2] C. M. WOODCOCK,[1] J. H. PARK,[3] R. L. PAUL,[4] K. S. BOO,[3] J. HARDIE,[2] and J. A. PICKETT[1,*]

[1]*Biological Chemistry Division, IACR-Rothamsted Harpenden, Herts., AL5 2JQ, United Kingdom*

[2]*Aphid Biology Group, Department of Biological Sciences Imperial College at Silwood Park Ascot, Berks., SL5 7PY, United Kingdom*

[3]*School of Agricultural Biotechnology Seoul National University Suwon 441-744, Korea*

[4]*School of Chemistry Cantock's Close, University of Bristol Bristol, BS8 1TS, United Kingdom*

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Abstract—The enantiomerically pure diastereoisomers (1*R*,4*S*,4a*R*,7*S*,7a*R*)-(**1**) and (1*R*,4*R*,4a*R*,7*S*,7a*R*)-dihydronepetalactol (**2**) were synthesized diastereoselectively from a renewable resource, (4a*S*,7*S*,7a*R*)-nepetalactone (**3**), isolated as the main constituent of the essential oil of the catmint plant *Nepeta cataria*. The stereochemistry of the compounds was determined by NMR spectroscopy and X-ray crystallography, and the compounds were identified, respectively, as neomatatabiol and isoneomatatabiol, natural products from *Actinidia polygama*, for which the lactol stereochemistry was previously incompletely defined. Compound **1** was found to catch significant numbers of three species of lacewing in the field: in Korea, *Chrysopa cognata*, and in the United Kingdom, *Nineta vittata* and most notably *Peyerimhoffina gracilis*. All species caught in significant numbers were found more frequently in traps releasing **1** than **2**, while more *C. cognata*, *C. formosa*, and *C. phyllochroma* were found in traps releasing (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**4**). The catch of *P. gracilis* with **1** is of particular interest as this lacewing has only recently been recorded in the United Kingdom.

*To whom correspondence should be addressed. E-mail: john.pickett@bbsrc.ac.uk

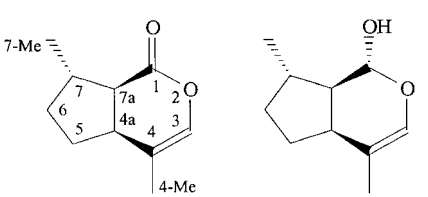
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Where sexed, the lacewings of all species trapped were found to be male, implying a possible pheromonal role for these or structurally related compounds.

Key Words—*Nepeta cataria*, *Chrysopa cognata*, *Chrysopa formosa*, *Chrysopa phyllochroma*, *Nineta vittata*, *Peyerimhoffina gracilis*, lacewing, pheromone, dihydronepetalactol, neomatatabiol, isoneomatatabiol.

INTRODUCTION

The cyclopentanoids (4a*S*,7*S*,7a*R*)-nepetalactone (**3**) and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**4**) (Figure 1) comprise the sex pheromones of a number of pest aphids (Aphididae), either as single compounds or as species-specific blends (Dawson et al., 1987, 1989). Aphid species including *Myzus persicae*, *Aphis fabae*, *Acyrthosiphon pisum*, *Sitobion fragariae*, *Tuberocephalus momonis*, and *Rhopalosiphum padi* have been trapped in the field using synthetic samples of these pheromone components (Dawson et al., 1990; Hardie et al., 1997; Boo et al., 2000). In addition, predatory species of lacewing such as *Chrysopa cognata* (Chrysopidae) are caught in traps releasing the same compounds, leading to the suggestion that, just as many aphid species use similar sex pheromones, lacewing predators may be using these compounds as kairomones in locating prey (Boo et al., 1998). Earlier literature reports revealed that the lacewing *Chrysopa septempunctata* was attracted to the vine *Actinidia polygama* (Actinidiaceae), a plant that generates two dihydronepetalactols previously named neomatatabiol and isoneomatatabiol (Hyeon et al., 1968), structurally related to **3** and **4**. The present work aimed to synthesize neomatatabiol and isoneomatatabiol and to determine unambiguously their stereochemistry. Studies were then undertaken to compare the potency of the aphid sex pheromone components **3** and **4** with the dihydronepetalactols related stereochemically to **3**, and to correlate lacewing catches in the field with the lactol stereochemistry not reported previously.



3 **4**

FIG. 1. Structures of aphid sex pheromone components (4a*S*,7*S*,7a*R*)-nepetalactone (**3**) and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**4**).

METHODS AND MATERIALS

Experimental Chemistry

NMR spectra were obtained using the Bruker 500 Avance NMR spectrometer. GC-MS analysis employed the VG-Autospec (Fisons Instruments) with a siloxane column (50 m × 0.32 mm ID; 0.52 μm) operated from 30°C to 250°C at 5°C/min; EI at 70 EV, 250°C. GC analysis was on an HP-5 siloxane column (30 m × 0.32 mm ID; 0.25 μm) operated from 40°C to 150°C at 5°C/min and from 150°C to 250°C at 10°C/min. Compounds eluted at 16.42 (**2**), 16.93 (**1**), 31.08 (**11**), and 31.44 min (**10**).

(*1*R,*4*S,*4a*R,*7*S,*7a*R)*-Dihydronepetalactol (**1**).* The essential oil from *N. cataria* (10.0 g, 60.2 mmol) was dissolved in ethanol (250 ml) under a hydrogen balloon with 10% palladium on charcoal (600 mg) as a catalyst. The mixture was stirred overnight, filtered, and the solvent removed *in vacuo*. A portion of the crude product (2.0 g, 11.9 mmol) was dissolved in dry THF (30 ml) at –78°C under nitrogen. DIBAL (13.1 ml, 1 M in hexanes) was added slowly and the mixture warmed to room temperature over 1 hr. The reaction was quenched without acidification, extracted with diethyl ether, and the combined organic extracts were dried ($MgSO_4$), filtered, and concentrated *in vacuo*. Purification by flash column chromatography (30% diethyl ether in petroleum ether) and recombination of the purest fractions gave **1** as a clear colorless oil (1.02 g, 50%). $[\alpha]_D^{20} - 1.1^\circ$ ($c = 9.0$, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.41 (1H, d, $J = 9.1$ Hz, H-1), 3.66 (1H, dd, $J = 5.2$, 11.8 Hz, *pro-R* H-3), 3.28 (1H, t, $J = 11.8$ Hz, *pro-S* H-3), 3.02 (1H, br s, OH), 2.25 (1H, m, H-4a), 2.14 (1H, m, H-4), 2.06 (1H, m, H-7), 1.98 (1H, m, *pro-R* H-6), 1.59 (1H, m, *pro-R* H-5), 1.46 (1H, m, H-7a), 1.44 (1H, m, *pro-S* H-5), 1.17 (1H, m, *pro-S* H-6), 1.00 (3H, d, $J = 6.9$ Hz, 7-CH_3), 0.80 (3H, d, $J = 7.1$ Hz, 4-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 97.1 (C1), 67.3 (C3), 53.2 (C7a), 41.7 (C4a), 34.5 (C7), 32.3 (C6), 30.1 (C4), 23.9 (C5), 22.4 (7Me), 14.7 (4Me); $m/z = 170$ (0.16%, M^+), 81 (100%), 67 (96), 41 (77), 137 (65), 39 (58), 95 (55), 109 (50), 79 (44), 152 (42, M^+-18), 82 (40), 27 (39), 110 (37), 107 (36), 55 (36), 53 (34), 43 (26), 77 (26), 29 (25).

*(1*R,*4*R,*4a*R,*7*S,*7a*R)*-Dihydronepetalactol (**2**).* The essential oil from *Nepeta cataria* (10.0 g, 60.2 mmol) was dissolved in methanol (100 ml) at 0°C. A solution of sodium borohydride (2.51 g, 66.2 mmol) in ice cold methanol (100 ml) was added to the mixture by cannula, and the reaction mixture was stirred until complete. The pH was adjusted to 7 after the addition of water (250 ml), and the mixture was extracted with diethyl ether. The combined organic extracts were washed with water, dried ($MgSO_4$), and concentrated *in vacuo*. A portion of the crude mixture (3.0 g, 17.9 mmol) was dissolved in ethanol (75 ml) under a balloon of hydrogen with 10% palladium on charcoal (200 mg) as a catalyst. The mixture was stirred overnight, filtered, and the solvent evaporated to yield a colorless oil containing

a 91:9 mixture of diastereoisomers (2.91 g, 96%). Purification of 2.91 g of the mixture by flash column chromatography (30% ether in petroleum ether) and recombination of the purest fractions gave **2** as a colorless oil (1.85 g, 64%). $[\alpha]_D^{20} + 9.4^\circ$ ($c = 6.2$, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.12 (1H, br s, H-1), 4.17 (1H, br s, OH), 3.60 (1H, t, $J = 11.0$ Hz, *pro-R* H-3), 3.42 (1H, dd, $J = 4.7$, 11.3 Hz, *pro-S* H-3), 2.00 (1H, m, H-7), 1.92 (1H, m, *pro-R* H-6), 1.88 (1H, m, H-4a), 1.68 (1H, m, *pro-R* H-5), 1.52 (1H, m, *pro-S* H-5), 1.46 (1H, m, H-4), 1.44 (1H, m, H-7a), 1.19 (1H, m, *pro-S* H-6), 1.01 (3H, d, $J = 6.3$ Hz, 7-CH_3), 0.79 (3H, d, $J = 6.6$ Hz, 4-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 92.8 (C1), 64.1 (C3), 51.1 (C7a), 41.5 (C4a), 33.6 (C7), 31.5 (C6), 30.9 (C4), 27.9 (C5), 20.0 (7Me), 15.5 (4Me); $m/z = 170$ (0.4%, M^+), 67 (100%), 81 (91), 137 (78), 41 (76), 95 (62), 39 (55), 109 (45), 152 (42, M^+-18), 79 (42), 27 (37), 110 (34), 53 (33), 55 (33), 82 (32), 107 (31), 77 (26).

*(1*R*,4*S*,4a*R*,7*S*,7a*R*)-Dihydronepetalactol Dimer (**10**).* Isomer **1** (420 mg, 2.47 mmol) was dissolved in diethyl ether (15 ml) and 6 N HCl (9 drops) and stirred overnight. The solvent was removed by rotary evaporation, and the residue was purified by flash column chromatography (10% diethyl ether in petroleum ether) to yield **10** as a clear colorless oil (300 mg, 75%). $[\alpha]_D^{20} - 25.5^\circ$ ($c = 10.2$, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.51 (1H, d, $J = 7.4$ Hz, H-1), 3.65 (1H, dd, $J = 4.9$, 11.3 Hz, *pro-R* H-3), 3.27 (1H, t, $J = 11.3$ Hz, *pro-S* H-3), 2.25 (1H, m, H-4a), 2.22 (1H, m, H-4), 1.96 (1H, m, H-7), 1.94 (1H, m, *pro-R* H-6), 1.57 (2H, m, *pro-R* H-5, H-7a), 1.43 (1H, m, *pro-S* H-5), 1.12 (1H, m, *pro-S* H-6), 0.99 (3H, d, $J = 6.6$ Hz, 7-CH_3), 0.80 (3H, d, $J = 6.6$ Hz, 4-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 98.1 (C1), 66.9 (C3), 50.6 (C7a), 40.9 (C4a), 35.0 (C7), 32.9 (C6), 29.6 (C4), 24.3 (C5), 21.8 (7Me), 14.8 (4Me); $m/z = 322$ (0.012%, M^+), 81 (100%), 67 (83), 137 (80), 41 (76), 95 (71), 109 (58), 39 (55), 152 (51), 70 (49), 153 (47), 107 (47), 110 (46), 27 (39), 55 (37), 53 (36), 93 (31), 77 (28), 43 (27).

*(1*R*,4*S*,4a*R*,7*S*,7a*R*)-Dihydronepetalactol Dimer (**11**).* Isomer **2** (2.41 g, 14.2 mmol) was dissolved in a mixture of diethyl ether (100 ml) and 6 N HCl (4 ml) and stirred overnight. The solvent was removed by rotary evaporation and the crude residue purified by flash column chromatography (10% diethyl ether in petroleum ether) to yield **11** as a white solid (1.76 g, 77%). Mp 110–113°C; $[\alpha]_D^{20} - 39.1^\circ$ ($c = 5.8$, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.02 (1H, br s, H-1), 3.36 (1H, dd, $J = 4.9$, 11.0 Hz, *pro-S* H-3), 3.29 (1H, t, $J = 11.0$ Hz, *pro-R* H-3), 2.01 (1H, m, H-7), 1.92 (1H, m, *pro-R* H-6), 1.79 (1H, m, H-4a), 1.68 (1H, m, *pro-R* H-5), 1.50 (1H, m, *pro-S* H-5), 1.44 (1H, m, H-4), 1.37 (1H, m, H-7a), 1.15 (1H, m, *pro-S* H-6), 1.00 (3H, d, $J = 6.4$ Hz, 7-CH_3), 0.75 (3H, d, $J = 6.4$ Hz, 4-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 92.7 (C1), 64.4 (C3), 50.8 (C7a), 41.8 (C4a), 33.6 (C7), 31.6 (C6), 31.1 (C4), 27.9 (C5), 19.9 (7Me), 15.4 (4Me); $m/z = 322$ (0.001%, M^+), 67 (100%), 137 (97), 81 (96), 41 (77), 95 (73), 153

(59), 39 (59), 109 (51), 152 (50), 79 (49), 110 (43), 107 (39), 27 (37), 53 (35), 55 (34), 77 (29), 93 (28), 91 (26).

X-Ray Crystallography

Crystals of **11** suitable for single crystal X-ray structure determination were grown by recrystallization from hexane. A single crystal of **11** was mounted on a glass fiber using high-vacuum grease. X-ray measurements were made with a Bruker SMART CCD area-detector diffractometer with Mo-K_α radiation ($\lambda =$ 0.71073 Å); structure solution SHELXTL program system version 5.1, 1998.

*Crystal Data for **11**.* $C_{20}H_{34}O_3$; $M = 322.47$, orthorhombic, space group *P2(1)2(1)2(1)*, $a = 8.7397(13)$ Å, $b = 9.4463(14)$ Å, $c = 22.898(3)$ Å, $U =$ 1890.4(5) Å^3, $Z = 4$, $D_c = 1.133$ Mg/m^3, μ(Mo-K_α) 0.074 mm^{-1}, $F(000) =$ 712, $T = 173(2)$ K, 3336 independent reflections with $2\theta < 50^\circ$. Refinement of 212 parameters converged at final $R_1 = 0.0434$, $wR_2 = 0.0806$.

Field Trapping in Korea

Field trapping experiments were carried out at the Suwon Agricultural Campus of Seoul National University and in the campus arboretum during July and August 2000. Wing trap dispensers (IMP Technology Inc.) and rubber septa (Sigma) were used. The traps were mounted 1.5 m above the ground and 2 m apart. The traps were checked and rotated 90° at 06:00 hr every morning. Analysis of trap catches was carried by using the GLIM statistics program (Crawley, 1993). The analysis was carried out declaring Poisson errors, with the catch for each replicate treated as independent.

Field Trapping in the United Kingdom

Lacewings were caught in clear water traps made of 14-cm-diam. × 2-cm-deep Petri dishes filled with a 1% clear odorless detergent solution. The traps were placed 2 m apart on 1.1-m-high poles. The volatiles were located in the center of the Petri dish above the detergent level, in dispensers made of amber glass vials [Chromacol 08-CPV(A)] with 1-mm holes drilled in the plastic lids. The vials were suspended below plastic shields to prevent rain entering and affecting release rates. Vials contained either the test compound (10 mg) in diethyl ether (50 μl) or ether alone. Traps were located at four woodland edge sites at Silwood Park, Ascot, Berkshire, with 16 traps at each site. In each trial, there were two traps for each treatment at each site. Traps were distributed so that identical treatments did not occur in adjoining traps. During the trial, which was run between May and August 2000, traps were rotated every four to five days. Traps were visited every two to three days, when all trapped lacewings were removed, identified, and

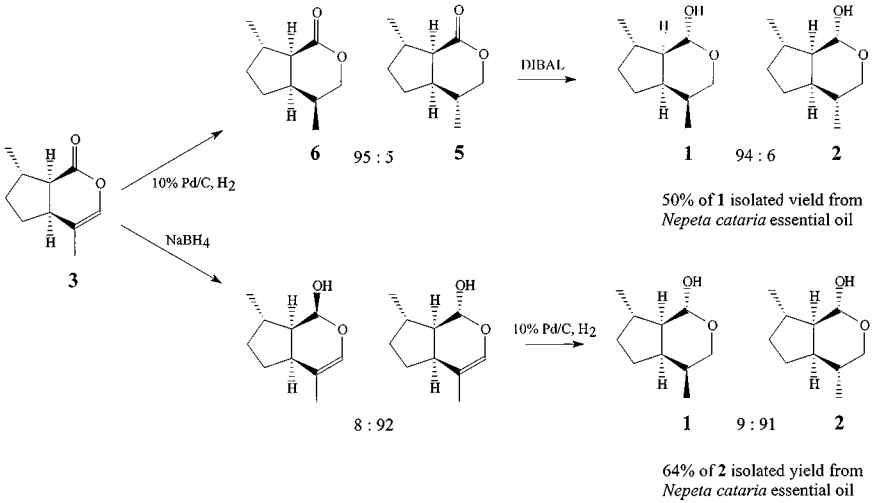
counted, and the solution in the traps was replaced. Analysis of water trap catches was carried with the GLIM statistics program (Crawley, 1993), with the trap totals for each treatment from each site being averaged, and each site treated as a block. The analysis was carried out declaring Poisson errors, with each site treated as an independent replicate.

Electrophysiology

Live adult male *Peyerimhoffina gracilis* were collected at Silwood Park, from traps made from clear plastic drinking bottles, during February and March 2001. Electroantennograms (EAGs) were obtained from excised antennae ($N = 7$), using Ag–AgCl glass electrodes filled with a saline solution. The signals generated by the antenna were passed through a high impedance amplifier (Syntech UN-06, Hilversum, The Netherlands), and data storage and processing were carried out with a PC-based interface and customised software package (Syntech). The system for stimulus delivery employed a filter paper strip in a disposable Pasteur pipet cartridge. Samples of test solutions (1 mg/ml in hexane, 10 μl applied) were applied to filter paper strips, and the solvent was allowed to evaporate for 30 sec before the paper strip was placed in the cartridge. A 2-sec stimulus (1 liter/min) was delivered into a purified airstream flowing continuously over the preparation. Results were expressed as a percentage of the response to a standard application of (*E*)-2-hexenal at the same concentration, and analysed for significant differences by Student's *t* test.

RESULTS AND DISCUSSION

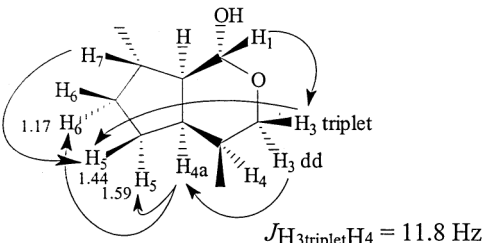
Synthesis and Structure Elucidation. (4a*S*,7*S*,7a*R*)-Nepetalactone **(3)** is found in the essential oil of many species of the *Nepeta* genus (Regnier et al., 1967; Eisenbraun et al., 1980; De Pascual Teresa et al., 1987) but, for high yield and purity, is obtained from *N. cataria* commercially by our collaborators (Botanix Limited) at approximately 90% of **3**. The essential oil was used here without further purification for the synthetic work. Compounds **1** and **2** were synthesized diastereoselectively from **3**, either by hydrogenation followed by lactone reduction in the case of **1**, or by lactone reduction followed by hydrogenation for **2**. Reduction of **3** with 10% palladium on carbon gave a 95:5 ratio of two products by GC, determined to be dihydronepetalactones by GC-MS (Scheme 1). The major component did not have the same spectroscopic properties as that reported for (4*R*,4a*R*,7*S*,7a*R*)-dihydronepetalactone (**5**) (De Pascual Teresa et al., 1987), but full characterization and stereochemical assignment by NMR spectroscopy was consistent with (4*S*,4a*R*,7*S*,7a*R*)-dihydronepetalactone **6**, formed by reduction from the less sterically hindered face. Interestingly, this is the opposite face selectivity to that reported when palladium on strontium carbonate is used as a



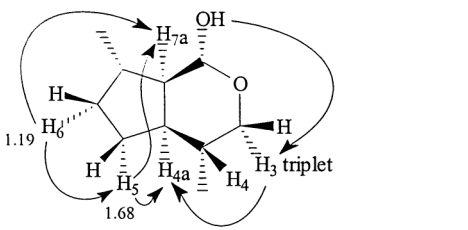
SCHEME 1. Divergent diastereoselective synthesis of **1** and **2** from (4a*S*,7*S*,7*R*)-nepetalactone (**3**).

hydrogenation catalyst (Regnier et al., 1967). In our hands, however, reduction with 2% $Pd/SrCO_3$ gave a different result from Regnier et al. (1967) and yielded the same major isomer **6** in a **6:5** ratio of 96:4 by GC. The crude mixture obtained from reduction with 10% palladium on carbon was then further reduced with DIBAL, and the crude product was analyzed by GC to reveal a 94:6 ratio of isomers **1:2**. By altering the order of chemical transformation, it was possible to synthesize **2** diastereoselectively. Borohydride reduction of the essential oil gave diastereomeric nepetalactols in the ratio 92:8, the stereochemistry of which was previously elucidated by X-ray crystallography of a derivative (Dawson et al., 1989). Hydrogenation of this crude mixture then gave a quantitative 91:9 mixture of **2:1** by GC. The diastereoselectivity of hydrogenation of nepetalactone can be explained on steric grounds, but, after borohydride reduction, the face selectivity of hydrogenation is reversed with comparable selectivity. This phenomenon has been observed previously when hydrogenation of *O*-ethoxyethyl protected (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**4**) was reported to occur with the same facial selectivity (Kigawa et al., 1992).

The relative stereochemistry of compounds **1** and **2** was determined by 1D gradient NOE spectroscopy (GOESY) (Figure 2) (Stonehouse et al., 1994). In the case of isomer **1**, the H-1 proton gives a NOE to the triplet at H-3 that, in turn, shows a NOE with a proton at H-5. Due to the known *cis* ring junction of **3**, this is only possible if H-1, H-3 (triplet), and the H-5 proton are all on the same upper face. The other H-3 proton, a doublet of doublets, shows a NOE to proton H-4a that is



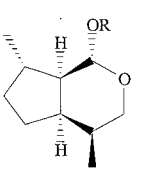
1



2

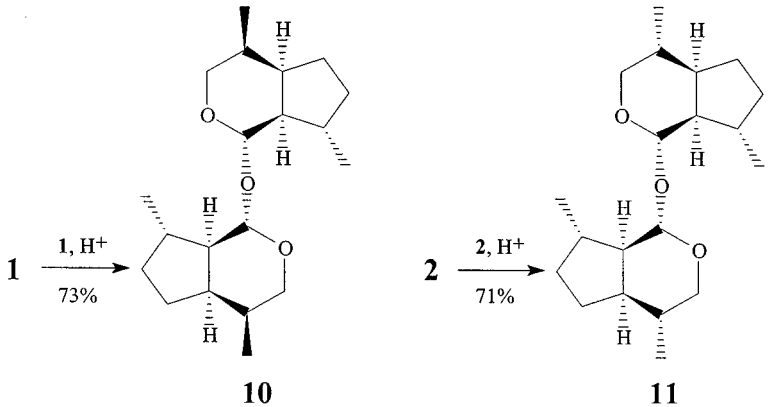
FIG. 2. Assignment of the stereochemistry of (1*R*,4*S*,4a*R*,7*S*,7a*R*)-dihydronepetalactol (**1**) and (1*R*,4*R*,4a*R*,7*S*,7a*R*)-dihydronepetalactol (**2**), showing the structurally revealing GOESY results.

known to be on the back face, verifying the structure. The methyl stereochemistry at C-4 is inferred by the large *trans* diaxial coupling constant of 11.8 Hz between the H-3 triplet and H-4. H-4a also shows a NOE to H-6 (1.17 ppm) and H-5 (1.59 ppm) and finally, a NOE from H-7 to H-5 (1.44 ppm) completes the assignment of the C-5 and C-6 protons. Several derivatives were made of **1** in an attempt to produce crystals for X-ray crystallography (Figure 3). None of compounds **7**–**9** was suitable for X-ray diffraction, but all showed the same NOEs between protons H-1, H-3 (triplet), and H-5, suggesting the same stereochemistry as presented in Figure 3. To ensure that the basic conditions used in generating **7**–**9** (treatment



7 : R = 3',5'-dinitrobenzoyl
8 : R = *p*-bromobenzoyl
9 : R = 3',5'-dinitrobenzyl

FIG. 3. Derivatives of **1** synthesized, which all revealed the same stereochemistry by GOESY spectroscopy.



SCHEME 2. Acid catalyzed dimerization of **1** and **2**.

of **1** with *n*-BuLi in anhydrous solvent followed by the acyl or alkyl chloride) was not epimerizing the lactol center, **7** was saponified in basic conditions and **1** reisolated with no lactol isomerization. An attempt to epimerize the lactol center of **1** using acid conditions was also made but instead of epimerization, **10** was isolated in good yield (Scheme 2). Infrared analysis of compound **10** showed it did not contain a hydroxyl group, and with one set of NMR peaks, it was characterized as a symmetric dimer. A very weak parent ion at *m/z* 322 was detected by electron impact mass spectroscopy. The ion at *m/z* 153 could be rationalized by loss of the $C_{10}H_{17}O_2$ group and the ion at *m/z* 152 by loss of dihydronepetalactol, analogous to loss of water from the monomeric compounds **1** and **2**. Again, NOE studies revealed the same relative stereochemistry as **1**, demonstrating the 1*R* stereochemistry to be the thermodynamically stable configuration as the reaction mechanism must involve equilibrium of **1** with an oxonium ion.

Despite purified **2** giving a single peak by three different GC columns (HP-1, HP-5, and β-cyclodextrin), its NMR spectrum in $CDCl_3$ showed it to be a mixture of two isomers, in a ratio of 90:10. GOESY studies on the major isomer **2** (Figure 2) revealed NOEs between the lactol OH and the H-3 triplet, which, in turn, gave a NOE to H-4a. As the ring junction stereochemistry is known, H-4a, H-3 (triplet), and the lactol OH must all be on the same back face. The stereochemistry at C-4 was assigned using the *trans* diaxial coupling of 11.0 Hz between H-3 (triplet) and H-4. Full assignment of the C-5 and C-6 protons was completed as the H-6 proton at 1.19 ppm shows a NOE to H-7a and the H-5 proton at 1.68 ppm, which in turn also relaxes H-4a and H-7a. GOESY studies on the minor isomer of the $CDCl_3$ solution of **2** revealed a NOE between H-1 and the H-3 triplet, demonstrating it to be the C-1 epimer. The treatment of **2** with acid generated a crystalline dimer, **11**. Mass spectrometry data for **11** were similar to those for **10**

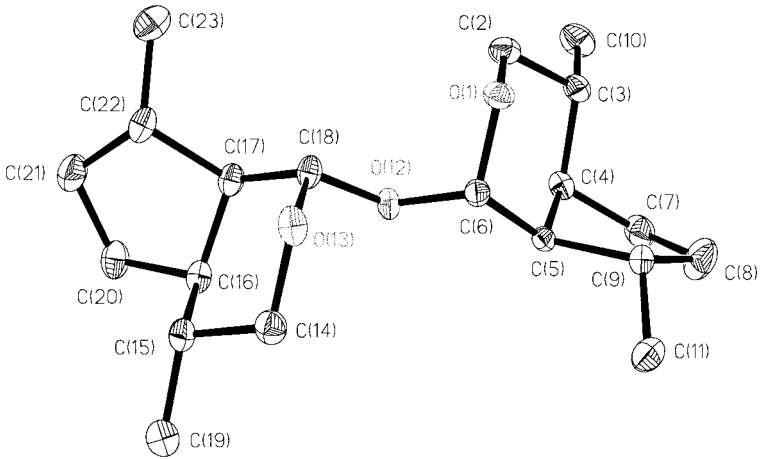


FIG. 4. Ortep diagram of **11**. Ellipsoids are drawn at the 30% probability level.

and crystals were obtained, suitable for X-ray crystallographic study (Scheme 2). The X-ray crystallographic results revealed the relative stereochemistry of **2** and based on the known chiral centers, stereochemistry at C-4 was verified as 4*R* and the thermodynamically stable lactol as 1*R* (Figure 4). By comparing the NMR data and synthetic methodology with the paper of Hyeon et al. (1968), structure **1**, (1*R*,4*S*,4a*R*,7*S*,7a*R*)-dihydronepetalactol, can be termed neomatatabiol and structure **2**, (1*R*,4*R*,4a*R*,7*S*,7a*R*)-dihydronepetalactol, can be termed isoneomatatabiol.

Field Results and Electrophysiology. Field trapping experiments in Suwon, Korea, and at Ascot, United Kingdom, caught several species showing varying preferences for the compounds described above. The initial study in Korea was performed between July 17 and 22, 2000. The traps releasing compound **1** caught significantly more *Chrysopa cognata* than did those releasing **2**. However, compound **4** was more effective than either, and also caught smaller but significant numbers of *Chrysopa formosa* and *Chrysopa phyllochroma* (Table 1). This study

TABLE 1. *Chrysopa* spp. CAPTURED IN SUWON, JULY 17–22, 2000[a]

Dispenser treatment	*C. cognata*	*C. formosa*	*C. phyllochroma*
1	171.3a	1.3ab	0.7a
2	63.7b	0.3b	0.3a
N. cataria extract containing **3**	20c	0b	0a
4	363.3d	17a	1.7a
Control	0e	0b	0a

[a] The insects were not sexed. Values are means. Means within a column followed by a different letter are significantly different at $P < 0.05$.

TABLE 2. *Chrysopa* spp. CAPTURED IN SUWON, AUGUST 2–6, 2000[a]

Dispenser treatment	*C. cognata*	*C. formosa* and *C. phyllochroma*
1	113a	1.3a
2	48b	0.7a
4	181.3c	3.7a
control	0d	0a

[a] All lacewings captured were male. Values are means. Means within a column followed by a different letter are significantly different at $P < 0.05$.

did not differentiate between male and female insects. The next study, which ran from August 2 to 6, 2000, caught fewer insects but they were all found to be male (Table 2). Although the catch means of *C. phyllochroma* (Table 1) and of *C. formosa* and *C. phyllochroma* (Table 2) for the different treatments did not differ significantly, analyses of variance showed the response to **4** to be significant at $P < 0.01$. The third trial studied the response of *C. cognata* to increasing release rates of **1** and **2** (Table 3).

In the United Kingdom, compounds **1** and **2** were initially tested for lacewing attraction in the field from May to July 2000 (Table 4). In this study, 11 specimens of *Nineta vittata* and 19 of *P. gracilis* were trapped, all of which were male. Further trials were performed during July and August 2000 (Table 5), with the addition of traps containing both **1** and **2** at equal concentrations and both at the same concentration as compounds in the other traps. Results showed compound **1** was

TABLE 3. *Chrysopa cognata* CAUGHT IN SUWON, AUGUST 6–10, 2000

Dispenser treatment	*C. cognata*/trap[a]
Compound **1**	
0.1 mg	8.7ab
1.0 mg	23.0a
10 mg	46.7c
50 mg	183.0d
Compound **2**	
2 0.1 mg	5.3b
2 1.0 mg	5.3b
2 10 mg	22.3a
2 50 mg	60.3c
Compound **4**, 10 mg	110.7e
Control	1.3b

[a] Values are means. means within a column followed by a different letter are significantly different at $P < 0.05$.

TABLE 4. MALE *Nineta vittata* AND *Peyerimhoffina gracilis* CAUGHT AT SILWOOD PARK, MAY–JULY 2000[a]

Dispenser treatment	*N. vittata*	*P. gracilis*
1	9	18
2	1	0
3	1	0
4	0	1
Control	0	0

[a] Catches with compound **1** are significant at $P < 0.001$.

highly effective at trapping lacewings compared to the other compounds tested and that isomer **2** did not influence the response to **1**. Of the 198 specimens of *P. gracilis* trapped in this study, only one was female.

Previous work (Hyeon et al., 1968) reported the compounds now defined as **1** and **2** to be attractants of adult male *C. septempunctata* and male *Chrysopa japonica*. We have demonstrated that the field response of adult male *P. gracilis* and *N. vittata* can be attributed to the 1*R*,4*S* stereochemistry of **1**. The preference of adult male *C. cognata* is for 1*R*,4*S* over 1*R*,4*R*, but the overall preference of *C. cognata*, *C. formosa*, and *C. phyllochroma* is for compound **4**.

Results of EAG analysis using male *P. gracilis* collected from the same trapping area showed compounds **1** and **2**, and the two aphid sex pheromone components, (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**4**) and (4a*S*,7*S*,7a*R*)-nepetalactone (**3**), to have significant activity (difference from control, $P < 0.001$), the first eliciting a significantly higher response than the latter three ($P < 0.05$) (Table 6). The results demonstrate that the antennae of *P. gracilis* show a strong response to all the compounds tested in the traps, but the largest EAG response to **1** correlates with the finding that this compound is the most active in the field.

TABLE 5. MALE *Peyerimhoffina gracilis* CAUGHT AT SILWOOD PARK, JULY–AUGUST 2000[a]

Dispenser treatment	*P. gracilis*
1	82
2	1
3	5
4	10
1 + **2**	95[b]
Control	4

[a] Catches with compound **1** and **1** + **2** are significant at $P < 0.001$ and differ from the control at $P < 0.05$.
[b] One female was also caught in this treatment.

TABLE 6. EAG RESPONSES OF MALE *Peyerimhoffina gracilis* TO TEST COMPOUNDS (1 mg/ml, 10 μl APPLIED), EXPRESSED AS PERCENTAGE OF RESPONSE TO (*E*)-2-HEXENAL AT THE SAME CONCENTRATION

Stimulus	Response[a]
1	266 ± 30.52a
2	160 ± 16.38b
3	175 ± 26.09b
4	185 ± 13.42b
Hexane control	47 ± 7.29

[a] Numbers followed by a different letter are significantly different at $P < 0.05$; all treatments are significantly different from the hexane control at $P < 0.001$ ($N = 7$).

The trapping of large numbers of *P. gracilis* was of interest for several reasons. This species was not known to be native to the U.K. and recent surveys at Silwood Park did not reveal its presence (Hollier and Belshaw, 1992, 1993; see Donato et al., 2001). In addition, during the period of trapping, no specimens of *P. gracilis* were caught in malaise traps or a suction trap present in the same area. Only in the traps releasing cyclopentanoids were large numbers caught. This species is believed to be relatively sedentary (Duelli, 1984) and to have a particular preference for conifer trees, which were not present in the immediate vicinity of the field sites at Silwood Park. Each of the four sites used in this trial was at least 20 m from a tree of a species known to support *P. gracilis* and three sites were approximately 100 m away. This suggests that either the species is more mobile and less constrained by habitat than is currently thought or that these compounds are effective over a considerable distance.

The presence of lacewings in traps releasing aphid sex pheromones and closely related cyclopentanoids may suggest a kairomonal role for these compounds in prey location, as has previously been proposed (Boo et al., 1998). Lacewings are polyphagous and feed on a number of insect species, including aphids. The fact that many aphid species use the same sex pheromone components would allow their use as indicators of aphid prey. Lacewing larvae are insectivorous predators but only a minority of species is predatory as adults; the majority feed on honeydew, nectar, and pollen. Of the species recorded in this study, adults of the genera *Nineta* and *Chrysopa* are insectivorous; *Peyerimhoffina* adults are not. The compounds tested here, therefore, elicit a response from both predatory and nonpredatory species, so it is unlikely that food location by adults is the cause of the observed activity.

It is possible that lacewings are sensitive to these compounds as an aid to the location of suitable oviposition sites. However, aphids only produce their sex

pheromones during the autumn, as part of the sexual stage in the life cycle that culminates in the production of overwintering eggs. In the United Kingdom, lacewings usually overwinter as pupae. Of the British species reported here, *N. vittata* is typically recorded as an adult from May to August (Plant, 1994), and so would not normally be exposed to the aphid sex pheromones. Although *P. gracilis* is present as an adult in the autumn, studies suggest that its oviposition is stimulated by increasing day length (Grimal, 1988). Therefore, it might be expected to utilize prey-produced kairomonal cues to locate suitable oviposition sites during the spring, rather than during the autumn months. If these compounds were functioning as prey-location kairomones, it would be expected that the numbers trapped would either show little sex bias or would be biased towards females looking to oviposit near a food source, as is the case with *Praon* parasitoids of aphids (Hardie et al., 1991). However, only male insects were detected in these trials, suggesting that these compounds, or analogous structures, may play a pheromonal role in intraspecies sexual communication and that their similarity to aphid sex pheromones may be simply incidental.

The possible role played by pheromones in intraspecific communication within the Chrysopidae, and the Neuroptera as a whole, is poorly understood. The only compound identified to date as a lacewing secretion is (*Z*)-4-tridecene, which has recently been identified from *Chrysoperla carnea* and appears to have defensive functions (Zhu et al., 2000). Although close-range communication in the Chrysopidae involves species-specific substrate-borne songs (Henry, 1979), it is thought that sex pheromones are involved in the initial mate location process (Walker et al., 1994). However, no sex pheromones or glandular sources for pheromones have been found. In the related Myrmeleontidae, some pheromones with a role in sexual communication are known, but these species exhibit a lek mating system and the pheromones eliciting a behavioral response are male-produced. Instead of functioning as sex pheromones, they appear to act as aggregation pheromones, with females responding to groups of males rather than to individuals (Yasseri et al., 1998). Although members of the Chrysopidae appear to be solitary and do not lek, a possible role for these compounds as aggregation pheromones cannot be discounted. In the Myrmeleontidae, different species utilize different blends of related compounds, providing species-specific signals (Baeckstrom et al., 1989; Bergstrom et al., 1992). From the results obtained here, in particular the differences in response shown to the compounds by the different *Chrysopa* species, it would seem possible that a similar pattern might be found in chrysopid lacewings. The demonstration that *P. gracilis* antennae respond strongly to all the cyclopentanoids tested, and the behavioral response of males of some species to more than one cyclopentanoid structure, is consistent with the possibility that these, or similar structures, may be natural pheromones.

The reasons for the appearance of *P. gracilis* in the United Kingdom may be speculated as being due to accidental release, its presence previously being

overlooked, wind-borne arrival, or range expansion as a consequence of global warming (Donato et al., 2001). The identification of cyclopentanoid monoterpenes as semiochemicals for a number of lacewings will facilitate monitoring the changing status of the new species and may allow manipulation of natural populations of these beneficial insects in integrated pest control strategies. Further research into the ecological and possibly pheromonal roles of these and related compounds is ongoing.

Supplementary Data—The CIF file for the crystal structure has been deposited at the Cambridge Crystallographic Centre, 12 Union Road, Cambridge CB2 1EZ, UK (www.ccdc.cam.ac.uk) from which it is available on request. The deposition number is CCDC 165880.

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ANNOUNCEMENT

19TH ANNUAL MEETING OF THE INTERNATIONAL SOCIETY OF CHEMICAL ECOLOGY AUGUST 3–8, 2002, HAMBURG

The 19th annual ISCE meeting will be held at the Department of Chemistry, University of Hamburg, Germany. The conference will begin on the evening of August 3, 2002 (Saturday) and end on the evening of August 7 (Wednesday). The meetings will be hosted by Wittko Francke.

There will be four symposia covering aspects of chemical ecology in symbiosis, molecular biology, insect-plant relationship, and pheromone chemistry.

Both oral and poster presentations are invited from participants. Contributed papers fitting to the symposia are particularly welcome.

Participants are encouraged to arrive on Saturday, August 3, and attend the Social Lecture given by Prof. Dr. Jerrold Meinwald followed by a mixer that evening. The concluding banquet will be held Wednesday evening, August 7.

Further Information may be obtained on the meetings web site http://isce2002.chemie.uni-hamburg.de

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IDENTIFICATION AND CLONING OF A FEMALE ANTENNA-SPECIFIC ODORANT-BINDING PROTEIN IN THE MOSQUITO *Culex quinquefasciatus*

YUKO ISHIDA,[1] ANTHONY J. CORNEL,[2] and WALTER S. LEAL[1,*]

[1]*Honorary Maeda-Duffey Laboratory*

[2]*Mosquito Control Research Laboratory, Department of Entomology, University of California Davis, Davis, CA 95616*

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Abstract—We have identified and cloned a cDNA encoding the first odorant-binding protein isolated from mosquitoes. The protein isolated from female antennae of *Culex quinquefasciatus* (CquiOBP) was not detected in legs (control tissue) or in antennal extracts from males, and showed mobility in native polyacrylamide gels similar to that of the pheromone-binding protein from *Bombyx mori.* The open reading frame of the cloned cDNA encoded a hydrophobic signal peptide (24 residues) and an acid mature protein (pI 5.5) of 125 amino acid residues (calculated molecular mass 14,504 Da). The transcript was detected by RT-PCR with antennal, but not with leg tissues. CquiOPB shared the highest amino acid identity with a product deduced from *Drosophila melanogaster* PBPRP-3 cDNA (58.6%), OBPs from scarab beetles (35%), and moths (28%). In addition, CquiOBP showed the hallmark of insect odorant-binding proteins, the six Cys residues.

Key Words—Odorant-binding protein, mosquito, *Culex quinquefasciatus*, Diptera, Culicidae.

INTRODUCTION

Female mosquitoes perceive the environment through semiochemicals, which they utilize for essential tasks, such as location of hosts (Davis and Bowen, 1994) and oviposition sites (Laurence and Pickett, 1982). To guide the mosquito behavior, these molecules must reach the olfactory receptors located in the dendrites, which are surrounded by a sensillar lymph. The transport of the hydrophobic semiochemicals through this aqueous environment is assisted by proteins, odorant-biding

* To whom correspondence should be addressed. E-mail: wsleal@ucdavis.edu

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proteins (OBPs), which solubilize the ligands, ferry them to their receptors, and protect the molecules from inactivation before their messages are delivered. Research has been conducted to gain a better understanding of the molecular basis of semiochemical perception in the cosmotropical nuisance mosquito *Culex quinquefasciatus* Say (Diptera: Culicidae). *Cx. quinquefasciatus* is a vector of bancroftian filariasis caused by the nematode *Wuchereria bancrofti*, a debilitating helminthic disease that afflicts 119 million people world-wide (Michael et al., 1996). Here, we report the first isolation, identification, and cDNA cloning of a dipteran OBP.

METHODS AND MATERIALS

Protein Extracts and Analytical Procedures. Three to five-day old adult mosquitoes were anesthetized on ice. Females were inseminated, but deprived of a blood meal. Antennae and control tissues (midlegs with tarsi removed) were dissected and homogenized in ice-cold glass homogenizers with 10 mM Tris-HCl, pH 8. Homogenized samples were centrifuged twice at 12,000 rpm for 10 min at 4°C. After concentration, the supernatants were analyzed by native polyacrylamide gel electrophoresis (15% PAGE) and stained with Coomassie blue R-250. For sequencing, proteins were electroblotted onto polyvinyl difluoride membranes, bands were cut, and N-terminal sequences were obtained on a Procise Protein Sequencing System (Applied Biosystems).

cDNA Cloning and RT-PCR. mRNA samples were prepared from 1,000 antennae and 200 midlegs of female *Cx. quinquefasciatus* using TRIzol (Invitrogen) and PolyATract mRNA Isolation Systems (Promega). cDNAs were synthesized by SMART RACE cDNA Amplification Kit (Clontech). *TAKARA LA Taq* (TAKARA) was used as Taq polymerase. To clone CquiOBP cDNA, we used the following primers: the degenerate primer 5′-GA(C/T)GC(A/C/G/T)GA(A/G)TA(C/T)-CC(A/C/G/T)CC(A/C/G/T)CC(A/C/G/T)GA-3′ was designed on the basis of N-terminal amino acid sequence of the isolated protein, GCGTTTTTTTTTTTTTT (3′-RACE), a UPM primer from the SMART RACE cDNA Amplification Kit and a gene-specific primer (GSP1; 5′-RACE), 5′-CCCCCTCCGGATACAGGCAACG-TTTTCCCA-3′, and GSP-2 (RT-PCR), 5′-CGAGGCCATCATTGAGTTCAGC-GACGGCAA-3′. Polymerase chain reactions (PCR) were carried out according to instruction manual of the SMART RACE cDNA Amplification Kit. PCR products were ligated into pBluescript SK(+) (Stratagene) and sequenced. Homology analyses were carried out using FASTA (http://www.ddbj.nig.ac.jp/). Alignment of amino acid sequences was drawn by MacVector (Oxford Scientific).

RESULTS AND DISCUSSION

Native polyacrylamide gel electrophoresis analysis of protein extracts from antennae and legs of *Cx. quinquesfasciatus* showed the occurrence of an

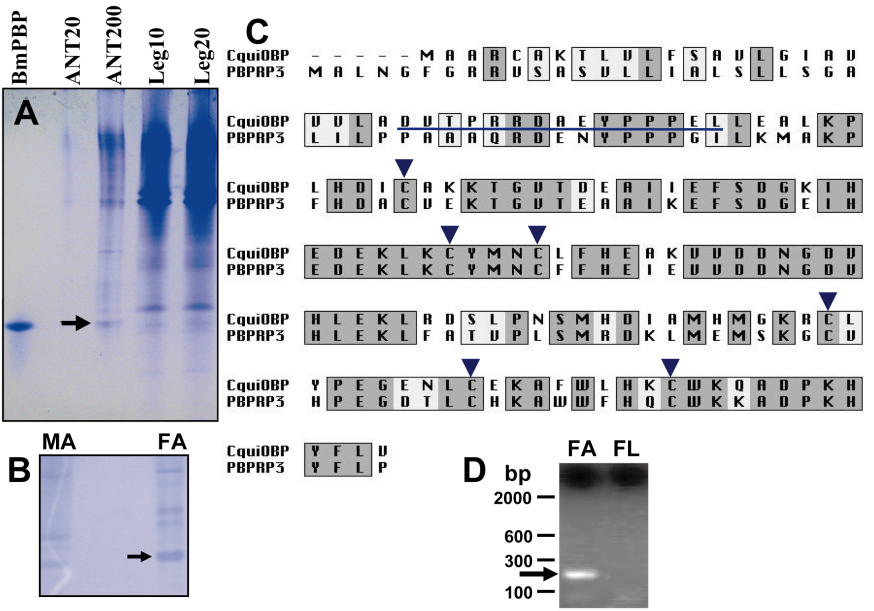


FIG. 1. Gel electrophoresis analysis and cDNA cloning data of the female- and antenna-specific protein from *Cx. quinquefasciatus.* A) Soluble proteins separated on a 15% native PAGE, stained with Coomassie blue. BmPBP, recombinant protein from the silkworm moth *B. mori* as a reference; ANT20 and ANT 200, extracts from 20 and 200 female antennae; Leg 10 and Leg20, extracts from 10 and 20 midlegs as control tissues. The second lane of the gel was left empty to avoid possible contamination from the recombinant protein. B) Comparison of protein profiles from male (MA) and female antennae (FA; 600 antennae of each sex), highlighting a female-specific protein (arrow). C) Sequence alignment of *Cx. quinquefasciatus* odorant-binding protein (CquiOBP) and a product deduced from *D. melanogaster* PBPRP-3 cDNA, which encodes a protein with the highest amino acid similarity in the database. The N-terminal sequence of the mature protein, obtained by Edman degradation, is underlined. Arrowheads show the six Cys residues, a hallmark of insect OBPs. The cDNA sequence has been submitted to the GenBank data base with accession number AF468212. D) cDNA fragment (arrow) amplified with gene-specific primers by using female antenna (FA) cDNA. Under the same conditions, PCR with female leg (FL) cDNA gave only primer dimers (bottom of the gel).

antenna-specific band (Figure 1A), which had a mobility similar to the pheromone-binding protein previously identified from the silkworm moth, *Bombyx mori* (BmPBP). Comparison of extracts from mosquito male and female antennae demonstrated that the antenna-specific protein was also specific to females (Figure 1B). We then transferred the protein from native gels to polyvinyl difluoride membranes to obtain its N-terminal amino acid sequence by Edman degradation.

The sequence (DVTPRRDAEYPPPEL) was proline-rich and showed significant amino acid identity to putative odorant binding proteins from *D. melanogaster* (Pikielny et al., 1994). The same protein was not detected in legs.

Our initial approach to clone the cDNA encoding the isolated female and antenna-specific protein was based on a degenerate primer designed according to the N-terminal amino acid sequence and an oligo (dT) to anneal to the poly(A) tail. The initial product amplified by PCR (3′-RACE) was used to prepare a gene-specific primer (GSP-1) so as to obtain the full length cDNA by 5′-RACE. The cloned cDNA consisted of 708 bp, with an open reading frame (ORF) of 450 bp, a 3′-noncoding region characterized by an AATAAA signal located 15 bases upstream of a poly(A) sequence. This cDNA encodes a peptide consisting of 149 amino acids, with the first 24 residues forming the signal peptide. The signal peptide was predicted by using von Heijne's criteria for eukaryotic signal peptides, confirmed by the data for the N-terminal sequence of the mature protein, and showed the expected hydrophobic region in a Kyte-Doolitle profile.

The mature protein encoded by this cDNA consisted of 125 amino acid residues (Figure 1C), with a calculated molecular mass of 14,504 Da, and pI of 5.5. The sequence showed the hallmark of odorant-binding proteins, six Cys residues at positions 26, 53, 57, 95, 104, and 113. In addition, prediction of the secondary structure by the Chou-Fasman method indicated that the protein is helical-rich (60.8%), which is another feature of insect odorant-binding proteins.

The tissue-specificity of the female protein was further investigated by RT-PCR by using two gene-specific primers to give a 199 bp fragment of the ORF. The expected cDNA fragment was amplified with female antenna, but not with leg template (Figure 1D). Because the protein is tissue-specific and shows remarkable similarity to previously identified odorant-binding proteins from insects, we suggest that this female-specific protein from *Cx. quinquefasciatus* is an odorant-binding protein (CquiOBP).

A database search using FASTA indicated that CquiOPB shared significant sequence similarity with putative odorant-binding proteins from *D. melanogaster*, whose native proteins have not been isolated to date. One of these gene products named PBPRP-3 (pheromone-binding protein-related protein) (Pikielny et al., 1994) showed the highest score (58.6% identity) (Figure 1C). The next highest scores were from OBPs from scarab beetles (34–35% identity) (Wojtasek et al., 1999; Peng and Leal, 2001; Deyu and Leal, 2002), whereas lepidopteran OBPs (Vogt et al., 1999) showed lower amino acid identities (<29%).

Various mosquito behaviors are mediated by volatile semiochemicals. It is also known that the semiochemical-detecting machinery is involved in the perception of the synthetic repellent DEET (Leal and Uchida, 1998; Costantini et al., 2001). Therefore, odorant-binding proteins may be invaluable tools for the screening of mosquito attractants and repellents. Given that only compounds that bind to OBPs are transported to the olfactory receptors, a throughput binding assay with

recombinant OBPs would allow the screening of a large number of compounds of potential application in mosquito trapping or repellents.

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POSTGENOMIC CHEMICAL ECOLOGY: FROM GENETIC CODE TO ECOLOGICAL INTERACTIONS[1]

MAY R. BERENBAUM[2,*]

[2]*Department of Entomology, 320 Morrill Hall*
University of Illinois
505 S. Goodwin
Urbana, Illinois 61801-3795

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Abstract—Environmental response genes are defined as those encoding proteins involved in interactions external to the organism, including interactions among organisms and between the organism and its abiotic environment. The general characteristics of environmental response genes include high diversity, proliferation by duplication events, rapid rates of evolution, and tissue- or temporal-specific expression. Thus, environmental response genes include those that encode proteins involved in the manufacture, binding, transport, and breakdown of semiochemicals. Postgenomic elucidation of the function of such genes requires an understanding of the chemical ecology of the organism and, in particular, of the "small molecules" that act as selective agents either by promoting survival or causing selective mortality. In this overview, the significance of several groups of environmental response genes is examined in the context of chemical ecology. Cytochrome P-450 monooxygenases provide a case in point; these enzymes are involved in the biosynthesis of furanocoumarins (furocoumarins), toxic allelochemicals, in plants, as well as in their detoxification by lepidopterans. Biochemical innovations in insects and plants have historically been broadly defined in a coevolutionary context. Considerable insight can be gained by defining with greater precision components of those broad traits that contribute to diversification. Molecular approaches now allow chemical ecologists to characterize specifically those biochemical innovations postulated to lead to adaptation and diversification in plant/insect interactions.

Key Words—Allelochemical, cytochrome P-450, environmental response gene, *Helicoverpa*, olfactory receptor, *Papilio*, substrate recognition site, xenobiotic response element, furanocoumarin, furocoumarin.

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* E-mail: maybe@uiuc.edu

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INTRODUCTION

The millenium bug, or Y2K bug, is the computer glitch that was a function of the fact that, in the 1950s, IBM engineers, to save memory, used only a two-digit year field; the two-digit field was anticipated to wreak havoc after December 31, 1999 (Anonymous, 2000). Such havoc conspicuously failed to materialize. Thus, an insect perhaps more worthy of the designation Y2K bug is *Drosophila melanogaster*, the vinegar or pomace fly. In March 2000, sequencing of the entire genome of *D. melanogaster* was completed and published, marking the first complete arthropod genome to be sequenced (Adams et al., 2000). This accomplishment may herald a new era within the field of chemical ecology. Since its inception, practitioners of the science of chemical ecology have been concerned mostly with small molecules. Admittedly, some reach impressive size; a case in point may be the extremely large-ring monocyclic polyazamacrolides produced in the defensive secretions of some coccinellid pupae. Ring sizes in some of these remarkable macrocycles can range from 24 to over 150 members (Schroeder et al., 2000), but substances with the cognomen "macromolecules" are only recently subjects for study by chemical ecologists. Protein serving as olfactory receptors and enzymes involved in allelochemical biosynthesis now routinely merit study, but even bigger molecules loom on the horizon.

In the postgenome era, DNA should be an increasing focus of attention even for those with an affection for small, tractable molecules. One reason is that DNA itself is a target site for many toxic natural products. The universal occurrence of DNA makes it a likely evolutionary target for broad-spectrum defense compounds, and the evolution of DNA must reflect in part interspecific interactions among organisms. B-DNA possesses quite a number of nucleophilic reactive sites that interact with natural products (Yang and Wang, 1999). Furanocoumarins (furocoumarins), e.g., benz-2-pyrone compounds produced by a handful of angiosperm plants primarily in the Apiaceae and Rutaceae, covalently bind to thymine and cross-link strands. Ecteinascidin from the marine tunicate *Ecteinascidia turbinata* binds covalently to guanine N-2; the fungal metabolite aflatoxin B1 attacks N-7 guanine. Duacarmycin, a *Streptomyces* product, and bleomycin/pepleomycin, from *Streptomyces verticillus*, induce DNA backbone cleavage. Daunorubicin, initially isolated from *Streptomyces caeruleorubidus*, and doxorubicin, produced by *Streptomyces peucetius*, are anthracycline drugs that can intercalate (as can triostin A and echinomycin, naturally occurring bisintercalator quinoxaline antibiotics from *Streptomyces echinatus* and related species); distamycin A, from *Streptomyces distallicus*, binds in the narrow minor groove associated with AT sequences in B-DNA, and actinomycin D from *Streptomyces* spp. binds to DNA duplexes and interferes with transcription.

Perhaps a more important reason for chemical ecologists to incorporate DNA into their studies is that one of the principal goals of postgenomic biology is to

ascertain gene function, and the function of many genes can be comprehended only within the context of chemical ecology, in particular, the function of those genes that can be considered "environmental response genes." Environmental response genes can be defined as those encoding proteins involved in interactions external to the organism, including interactions among organisms and between the organism and its abiotic environment. General characteristics of environmental response genes, particularly those involved in mediating interactions among organisms and thus allowing for reciprocal evolutionary responses, include (1) very high diversity, (2) proliferation by duplication events, (3) rapid rates of evolution, (4) occurrence in gene clusters, and (5) tissue- or temporal-specific expression. All of these characteristics are consistent with responses to evolutionary pressure emanating from a highly changeable external environment.

Among environmental response genes, then, are those encoding proteins involved in the manufacture, binding, transport, and breakdown of semiochemicals, the principal medium of communication for many species between the organism and its environment. Examples of environmental response genes are numerous. Chemical signals are characterized by producers and recipients, and each has its own set of genes (Table 1). These genes may have genome-level effects that are very different from, for example, the so-called "housekeeping genes." By the same token, the term in common use to contrast with housekeeping genes, i.e., "luxury genes" (e.g., Vinogradov, 1997), sells short the ecological and evolutionary contributions of genes allowing organisms to cope with environmental stress. The lack of correlation between genome size and number of genes (e.g., *Caenorhabditis elegans* at 97 Mb and 19,000 genes and *Drosophila melanogaster* with 180 Mb and 13,000 genes; http://www.ornl.gov/hgmis/faq/compgen.html) has led to the suggestion that evolution proceeds principally by altering patterns of gene expression, rather than by increasing the protein inventory. Environmental response genes may be an exception to the trend; new functions may be acquired by proliferation of new proteins, not just by changes in regulation and expression. Parallel evolution in both gene function and expression pattern may be the key to diversification in environmental response genes. In this overview, the significance of

TABLE 1. SEMIOCHEMICALS AND ASSOCIATED ENVIRONMENTAL RESPONSE GENES

Semiochemical	Producer	Receiver
Pheromone	Biosynthetic genes	Pheromone-binding protein genes
		Odorant receptor genes
		Pheromone-degrading enzymes
Allomone	Biosynthetic genes	Detoxification genes
	Transport protein genes	Transport protein genes
Kairomones	Biosynthetic genes	Odorant receptor genes
		Taste receptor genes

several groups of environmental response genes is examined in the context of chemical ecology.

The "chemical senses" by which organisms perceive information about their environments encompass olfaction, or perception of chemical stimuli in gaseous phase, and gustation (and other forms of contact chemoreception), perception of chemical stimuli as solutes in liquid media. Irrespective of organism, chemical signaling (not only externally but also internally) shares several features. Most, if not all, chemical signaling systems include a receptor that reacts with a signal molecule; many also involve a signal transducer and amplifier. Perireceptor phenomena associated with movement of signal molecules (binding, transport, and degradation) also share many conserved features across a wide range of taxa. Many of the same classes of molecules serve these functions across a variety of taxa.

GENES INVOLVED IN BINDING AND TRANSPORT OF SEMIOCHEMICALS

Taste Receptors. Molecular characterization of taste receptors has eluded investigators for a long time; molecular techniques provided a new and ultimately successful approach to this goal. Clyne et al. (2000) searched the *Drosophila melanogaster* genome to find a large family of putative odorant receptors, which they call the gustatory receptor family; the search algorithm identified proteins with characteristic structural properties (with sequence length around 380 amino acids, and regions encoding seven predicted transmembrane domains, suggestive of G-protein coupled receptors). At the time the study was done, 60% of the genome yielded 75 proteins in the family. Amino acid identity ranges from 34% to less than 10%, indicating tremendous sequence divergence. These gustatory receptors tend to be found in clusters; two large clusters contain four genes, whereas several other clusters contain two or three genes. Tissue specificity is high as well, in that 18 of the 19 characterized are expressed only in the labellum, the gustatory organ. Moreover, gene expression could not be detected in the labellum of a mutant, *pox neuro*, which lacks taste neurons.

Subsequent completion of the genome sequence of *D. melanogaster* unveiled many other members of this group of candidate taste receptors to bring the total to at least 57 genes (http://sdb.bio.purdue.edu/fly/aignfam/odorcpt3.htm). Clyne et al. (2000) concluded that "The large size of this protein family likely reflects the diversity of compounds that flies can detect." Alternatively, expression of individual genes is often restricted to subsets of gustatory neurons in developmentally different sense organs (Scott et al., 2001; Dunipace et al., 2001; Robertson, 2001). Given that so little is known of the chemical ecology of *D. melanogaster* in nature, identifying which compounds were likely selective agents in the evolution of this protein family will be a challenge.

Olfactory Receptors. Like gustatory receptors, olfactory receptors, characterized by multiple transmembrane domains, are extremely diverse within most genomes characterized to date. In the genome of the free-living nematode *Caenorhabditis elegans*, Robertson (1998) examined genes in the serpentine *str* (seven transmembrane receptor) family encoding seven-transmembrane G-protein coupled receptors related to ODR-10 diacetyl chemoreceptors (with a known function in detecting environmental chemicals) and identified 197 members of this group. The family shares a close relationship with another family of serpentine receptors, *srd* (serpentine receptor class D), with approximately 55 genes. In a subsequent study, Robertson (2000) discovered another family of candidate serpentine chemoreceptors: the *srh* (serpentine receptor class H) family, which contains 214 genes and 90 pseudogenes. As much as 6% of the genome may encode over 500 chemoreceptors, constituting up to 4% of *C. elegans* proteins, an indication of the importance of chemical senses to this soil-dwelling organism. Phylogenetic analyses suggest frequent gene duplication events, e.g., as many as 85 duplication events in family *srh* can be inferred to have occurred since *C. briggsae* and *C. elegans* differentiated. *C. elegans* is by no means unique in its reliance upon functional chemoreceptor genes; in mammals, olfactory receptors may number in excess of 1000, representing 1% of the gene complement.

Pheromone-Binding Proteins. Odorant-binding proteins in a wide range of organisms serve to transport generally hydrophobic environmental odorants to internal (olfactory) receptors. This is known to be a large family in *D. melanogaster*; Rubin et al. (2000) list 14 members of the family, and subsequent investigation has brought the number up to 39 (H. Robertson, personal communication). Among the odorant-binding proteins of insects are pheromone-binding proteins (PBP); these are involved in the protection and transport of pheromones from the external environment to internal receptors, and, like other members of the family, these proteins act generally as lipophilic ligand-transporting agents. In insects, they have been characterized in extracellular fluids bathing receptor cells in antennal hemolymph. Willett (2000) has conducted a study of pheromone-binding proteins within the genus *Choristoneura* in order to ascertain the forces driving diversification and differentiation in this group of proteins. *Choristoneura* is a genus of leaf-rolling caterpillars of economic importance, and a considerable amount of effort has been invested in characterizing the pheromones they produce. The putative ancestral pheromone is an acetate; the pheromone occurs as an alcohol in *C. parallela* and as an aldehyde in *C. fumiferana*. Willett (2000) was interested in finding evidence of selective impact on PBPs in association with changes in pheromone composition, specifically changes in the ratio of nonsynonymous to synonymous substitutions indicative of diversifying selection. Evidence of changes in PBPs corresponding to changes in pheromone composition was found in the case of *C. fumiferana*, with its novel aldehyde pheromone. However, other pheromone changes (e.g., chain

lengthening in *C. muriana* and a shift to an alcohol in *C. parallela*) are not reflected in changes in PBP.

BIOSYNTHETIC AND DETOXIFICATIVE PROTEINS

Over a quarter century of studies in chemical ecology have made clear the importance of semiochemical biosynthesis and detoxification to organisms across a wide range of taxa. Perhaps the best known group of environmental response genes are the cytochrome P-450s. Cytochrome P-450 monooxygenases (P-450s) comprise a large superfamily of heme-thiolate proteins that metabolize a wide range of both endogenous and exogenous hydrophobic compounds by incorporating oxygen into a functionalized product. This generalized reaction, converting lipophilic substrates into hydrophilic products, means that these enzymes display great versatility; reactions catalyzed by P-450s include such oxidative transformations as monooxygenations, NO-synthase type oxidations, dehydrogenations, and peroxidase-type oxidations and nonoxidative reactions such as dehydrase activity, nitric acid reductase activity, and isomerization (Mansuy, 1998). Thus, these enzymes participate in a tremendous variety of transformations, including both biosynthetic and detoxification reactions.

A standardized nomenclature based on levels of amino acid sequence identity is in use for this huge superfamily (Gonzalez and Gelboin, 1989). Sequences sharing over 40% identity are placed in a family (designated by a number); those sharing over 55% identity are placed in a separate subfamily (designated by a letter), and those sharing over 97% identity are recognized as allelic variants at a locus (designated by a number).

The number of P-450s represented in a genome varies with the organism. They make up one of the largest superfamilies in plants; the recent genome sequencing of *Arabidopsis thaliana* revealed over 280 P-450 genes (Paquette et al., 2000). In *Drosophila melanogaster*, 90 P-450s have been characterized, of which only seven are thought to be pseudogenes (Tijet et al., 2001a), and in *C. elegans* there are at least 60 P-450 genes (Gotoh, 1998). Gonzalez and Nebert (1990) suggested that P-450s began to diversify over 400 million years ago, concomitant with the colonization of terrestrial habitats by plants and herbivorous animals. The rapid diversification may have been a consequence of reciprocal selection pressures whereby plants biosynthetically evolve novel defense compounds, and insects (and other herbivores) overcame the erstwhile toxins with novel detoxification pathways. Multiple gene duplication events allowed P-450s to acquire new functions in the presence of new environmental stress factors while retaining ancestral metabolic capabilities. Thus, P-450s may well be the consummate environmental response genes, evolving and diversifying to a certain extent in the context of both interspecific and intraspecific interactions.

However, protein diversification is only one of several possible evolutionary mechanisms. P-450 expression is regulated in response to a wide range of factors, including developmental stage and exposure to both endogenous and exogenous substances (Tables 2 and 3). The majority of studies have focused on synthetic xenobiotics as inducers, whose efficacy was initially determined in studies of human P-450 (e.g., phenobarbital); their ecological significance is often difficult to interpret. The relative contributions of P-450 diversification and regulation of expression to the evolution of plant/animal interactions are unknown and will likely remain unknown until ecologically relevant inducers can be identified.

Despite the enormous significance of ecological interaction in understanding P-450s, the ecological function and evolutionary history of most P-450s have yet to be determined. The vast majority of studies of P-450s involve humans, particularly in the context of understanding drug metabolism and carcinogenesis (in that P-450s can act as activators of procarcinogens as well as detoxification enzymes) (e.g., Guengerich, 1998). Although there is no question that such work is of great importance to human health, it does not provide a great deal of insight into the evolutionary processes that led to P-450 diversification; humans are uniquely effective at circumventing the action of natural selection and adapting the environment to themselves rather than adapting themselves to the environment (Berenbaum, 1999). In organisms other than vertebrates, the emphasis in P-450 studies is generally on human-produced xenobiotics as substrates. Until recently, in plants, efforts in characterizing function have focused on elucidating the role of P-450s in herbicide tolerance (Werck-Reichart et al., 2000); similarly, most work with insect P-450s has been in the context of characterizing genes associated with resistance to synthetic organic insecticides (Scott, 1999).

The environmental context of P-450 evolution is perhaps best characterized in plants. Many P-450s have been characterized at the enzyme level and are known to participate in the biosynthesis of allelochemicals (Table 4, Fig. 1). As autotrophs, plants (with rare exceptions) are reliant upon biosynthesis, rather than sequestration, for the acquisition of secondary compounds. Moreover, as sedentary organisms that form the base of most food webs, they are subject to intense selection pressure from herbivores and have few options other than chemical or morphological defense for reducing the impact of herbivores (Berenbaum, 1995a). Thus, the allelochemical inventory of plants is staggering; over 40,000 compounds have been characterized. P-450-mediated transformations may well be involved in the biosynthesis of the majority of these compounds. Genome sequencing of plants with known secondary chemistry will facilitate elucidation of the function of many P-450 genes, as has already proved to be the case in *A. thaliana*; great strides have been made, e.g., in characterizing the genes governing glucosinolate biosynthesis (Table 4). Moreover, as in animals, P-450s play an important role in xenobiotic detoxification in plants; herbicides and other environmental toxins are metabolized by an array of P-450s (Table 4).

TABLE 2. TISSUE LOCALIZATION AND INDUCERS OF INSECT P-450 GENES IN INSECTS[a]

Isoform	Species	Tissues	Inducers	Ref.
CYP4C1	*Blaberus discoidalis*	FB	HTH	
CYP4D1	*D. melanogaster*	NR		
CYP4D10	*Drosophila mettleri*	NR	SCA, PB	Danielson et al. (1998)
CYP4E1	*D. melanogaster*	V	NR	
CYP4L1,2	*Manduca sexta*	MG, FB	NR	
CYP4M1	*M. sexta*	MG, FB	C, PB, U	
CYP4M2	*M. sexta*	MG, FB	Not induced	
CYP4M3	*M. sexta*	MG, FB	C, T, U	
CYP6A1	*M. domestica*	G, FB	PB, PBO, ET	
CYP6A2	*D. melanogaster*	FB, MG, MT	PB	
CYP6A4, 5	*M. domestica*	NR	NR	
CYP6A8,9	*D. melanogaster*	NR	BA	
CYP6B1	*Papilio polyxenes*	MG, FB, I	X, BG, A	Petersen et al. (2001)
CYP6B2	*Hellothis armigera*	MG, FB	PO, PBO, P	
CYP6B3	*P. polyxenes*	MG, FB	X, BG, A	Petersen et al. (2001)
CYP6B4	*P. glaucus*	MG	X, A, B, I	
CYP6B7	*H. armigera*	MG	Pinene	
CYP6B8	*H. zea*	MG, FB, RT	X, CA, IC, PB	X. Li et al. (2002)
CYP6B9	*H. zea*	MG	X, CA, IC, PB	X. Li et al. (2002)
CYP6B11	*P. canadensis*	MG	X	
CYP6B12	*P. glaucus*	MG	X	X. Li et al. (2001)
CYP6B16	*P. glaucus*	MG	X	X. Li et al. (2001)
CYP6B17	*P. glaucus*	MG	X	X. Li et al. (2001)
CYP6B18	*P. canadensis*	MG	X	X. Li et al. (2001)
CYP6B20	*P. glaucus*	MG	X	X. Li et al. (2001)
CYP6B21	*P. glaucus*	MG	X	X. Li et al. (2001)
CYP6B24	*P. glaucus*	MG	X	X. Li et al. (2001)
CYP6B25	*P. canadensis*	MG	X	X. Li et al. (2001)
CYP6B26	*P. canadensis*	MG	X	X. Li et al. (2001)
CYP6B27	*H. zea*	MG, FB	X, CA, IC, PB	X. Li et al. (2001)
CYP6B28	*H. zea*	MG, FB, RT, I	X, CA, IC, PB	X. Li et al. (2001)
CYP6D1	*M. domestica*	FB, PI, RT	PB, PBO, C	
CYP9A1	*H. virescens*	FB, G	NR	
CYP9A2	*M. sexta*	MG	U, IC, PB, T, X	Stevens et al. (2000)
CYP9A4	*M. sexta*	MG	C, X	Stevens et al. (2000)
CYP9A5	*M. sexta*	MG	X	Stevens et al. (2000)
CYP18	*D. melanogaster*	BW, G	EC	
CYP28A1	*D. mettleri*	NR	PB, SA	
CYP28A2	*D. mettleri*	NR	PB, SA, SCA	
CYP28A33	*D. nigrospiracula*	NR	PB, SCA	

[a] From Scott et al. (1998) unless otherwise indicated. Tissues: BW = body wall, FB = fatbody, I = integument, MG = midgut, G = gut, MT = Malpighian tubules, PI = proximal intestine, RT = reproductive tissue, V = viscera, NR = not reported. Inducers: A = angelicin, BA = barbital, B = bergapten, C = clofibrate, CA = chlorogenic acid, EC = ecdysone, ET = ethanol, HTH = hypertrehalosemic hormone, I = isopimpinellin, IC = indole-3-carbinol, N = nicotine, P = α-pinene, PB = phenobarbital, PBO = piperonyl butoxide, PO = peppermint oil, SA = senita alkaloids, SCA = saguaro cactus alkaloids, T = tridecanone, U = undecanone, X = xanthotoxin.

TABLE 3. KNOWN FUNCTIONS OF INSECT P-450S

Isoform	Species	Substrates	Ref.
CYP6A1	*Musca domestica*	farnesyl, geranyl, neryl methyl esters, aldrin, heptachlor	Andersen et al. (1997)
CYP6A2	*Drosophila melanogaster*	aflatoxin, 7, 12 dimethyl benz[*a*]anthracene 3-amino-1-methy1-5H-pyridoindole; aldrin, heptachlor, diazinon	Saner et al. (1996) Dunkov et al. (1997)
CYP6B1	*Papilio polyxenes*	bergapten, xanthotoxin, psoralen, isopimpinellin	Ma et al. (1994)
CYP6B3	*Papilio polyxenes*	xanthotoxin?	J. Chen (unpublished results)
CYP6B4	*Papilio glaucus*	bergapten, xanthotoxin, isopimpinellin, imperatorin	Hung et al. (1997)
CYP6D1	*Musca domestica*	benzo[*a*]pyrene, methoxyresorufin, deltamethrin, cypermethrin, chlorpyrifos	Scott (1999)

The *C. elegans* genome has revealed more than 60 P-450 genes (Gotoh, 1998), with gene structures "strikingly divergent within each group." The majority may deal with xenobiotic substrates—all of the P-450s characterized show relationships to mammalian CYP2, CYP3, and CYP4 subfamilies, which are involved in xenobiotic metabolism. Because the chemical ecology of *C. elegans* in nature is not particularly well characterized, elucidating the specific catalytic properties of these P450s will be challenging.

Drosophila genome sequencing has yielded 90 P-450s (Tijet et al., 2001a) (http://ag.arizona.edu/p450/insectlist.html), the function of the vast majority of which is unknown. Although much attention has been focused on P-450 participation in detoxification of such xenobiotics as insecticides, the majority of P-450s in *Drosophila melanogaster* may be involved in allowing this relatively generalized consumer of microbes and microbial fermentation products to deal with the wide range of microbial products, rather than plant allelochemicals, encountered. Furthermore, Danielson et al. (1998) have documented P-450 involvement in detoxification of cactus alkaloids in cactophilic *Drosophila* species; CYP families 4, 6, and 27 may be involved, as suggested by induction in the presence of potential cactus-derived substrates (Table 2).

Other degradative functions of P-450s may involve endogenous substrates involved in intraspecific communication; in a small expressed sequence tag project with *Manduca sexta*, Robertson et al. (1999) reported the presence of three P-450s that are candidate odorant degrading enzymes. P-450s are also involved in the biosynthesis of other intraspecific chemical signals such as pheromones and cuticular hydrocarbons, and such hormones as ecdysone and juvenile hormone (Feyereisen, 1999). Thus, given the multitude of functions of P-450s in insects,

TABLE 4. PLANT P-450S WITH KNOWN FUNCTIONS[a]

CYP number	Function	Reference
CYP51	obtusifoliol 14 *a*-demethylase	
CYP71A5	(3*S*)-cintronellol 10-hydroxylase	
CYP71A11	chlortoluron metabolism	
CYP71B7	7-ethoxycoumarin *O*-deethylase	
CYP71C	DIBOA biosynthesis	
CYP71C3v2	triasulfuron 5-hydroxylase	
CYP71D9	flavonoid-6-hydroxylase	Latunde-Dada et al. (2001)
CYP71D13	limonene-3-hydroxylase	Wust et al. (2001)
CYP71D18	limonene-6-hydroxylase	Wust et al. (2001)
CYP71E1	*p*-hydroxymandelonitrile synthesis	
CYP72A1	secologanin synthase	Irmler et al. (2000)
CYP73	cinnamate 4-hydroxylase	
CYP74A	alleneoxide synthase	
CYP74B	fatty acid hydroperoxidelyase	
CYP74C	fatty acid hydroperoxide lyase	Tijet et al. (2001b)
CYP75	flavonoid 3′, 5′-hydroxylase	
CYP76	7-ethoxycoumarin *O*-deethylase	
CYP78A1	fatty acid hydroxylase	
CYP79A1	tyrosine *N*-hydroxylase	
CYP79A2	phenylalanine *N*-hydroxylase	Wittstock and Halkier (2000)
CYP79B2	tryptophan to indole-3-acetaldoxime	Mikkelson et al. (2000)
CYP79F1	dihomomethionine to 5-methylthiopentanaldoxime	Hansen et al. (2001)
CYP80A1	berbamunine synthase	
CYP80B1v7	(*S*)-*N*-methylcoclaurine 3′-hydroxylase	
CYP80B1v2	fatty acid hydroxylase	
CYP81B1	fatty acid hydroxylase	
CYP81E1	flavonoid biosynthesis	
CYP83B1	indole-3-acetaldoxime hydroxylase	Bak et al. (2001)
CYP84	ferulate 5-hydroxylase	
CYP86A1	fatty acid *w*-hydroxylase	
CYP88A1	gibberellin biosynthesis	
CYP90A1	cathasterone 23-hydroxylase	
CYP90B	brassinosteroid biosynthesis	
CYP90C1	steroid hydroxylase	
CYP93A1	dihydroxypterocarpan 6*a*-hydroxylase	
CYP93B1	(2*S*)-flavanone 2-hydroxylase	
CYP93B2	flavone synthase II	Martens and Forkman (1999)
CYP93C1	8-hydroxyisoflavanone synthase	Latunde-Dada et al. (2001)
CYP93C2	2-hydroxyisoflavanone synthase	Akashi et al. (1999)
CYP94A1	cutin monomers synthase	
CYP701A3	ent-kaurene oxidase	
CYP703A1	fatty acid hydroxylase	

[a] From Ohkawa et al., 1999 unless otherwise indicated.

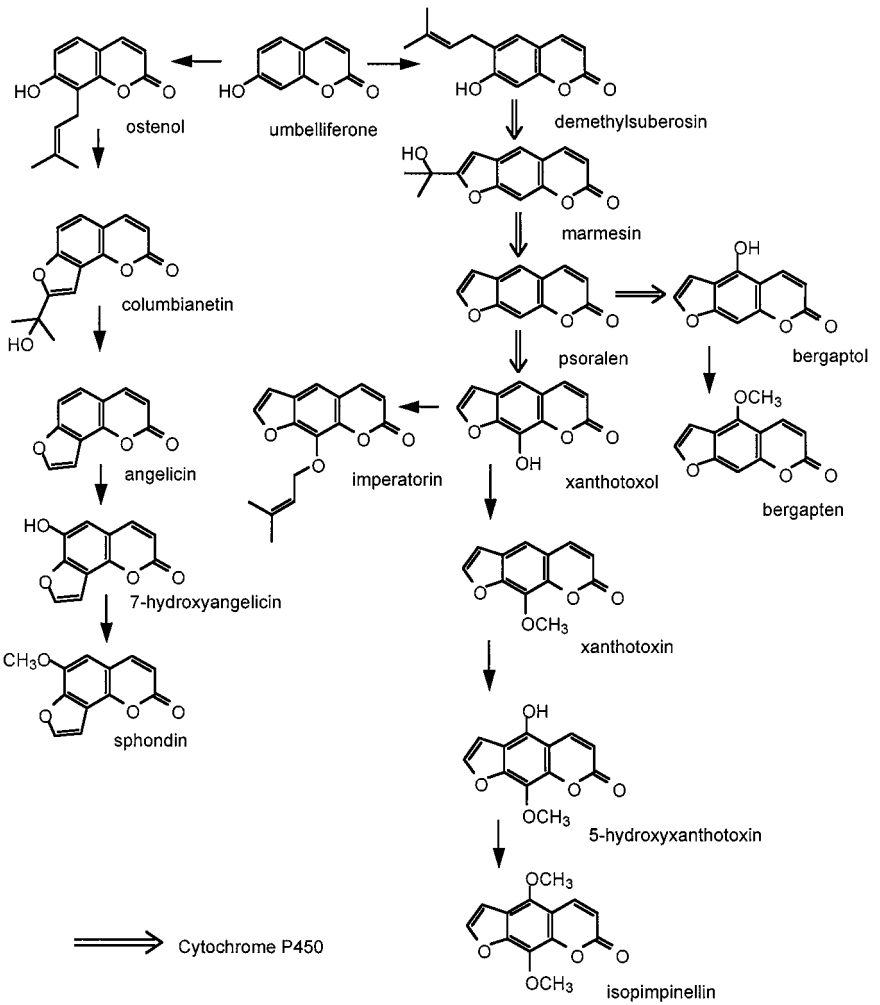


FIG. 1. Biosynthetic pathway leading to furanocoumarin production. Hollow arrows indicate steps known or suspected to be catalyzed by cytochrome P-450s. Angular furanocoumarin synthesis on left of figure, linear furanocoumarin synthesis on right of figure.

their diversity in at least the single insect genome characterized is not altogether surprising.

Like other environmental response genes, P-450s appear to arise and diversify via gene duplication and acquisition of new functions. Some are involved in metabolism of endogenous substrates, such as hormones, but extreme diversification appears to be a hallmark of the xenobiotic-metabolizing P-450s. To understand

the evolutionary diversification of P-450s, it is, therefore, important to identify the environmental chemicals (natural as well as synthetic) that act as selective agents promoting change.

P-450s in Plant/Insect Interactions—Furanocoumarins as Exemplars. The coevolutionary interactions between flowering plants and herbivorous insects (collectively constituting over half of all terrestrial species) are thought to have generated much of the terrestrial diversity on the planet. Ehrlich and Raven (1964) suggested that the evolution of biochemical novelty leads to speciation in angiosperms and that subsequent biochemical innovations in detoxification lead to speciation in herbivores associated with those plants: "If recombination or mutation appeared in a population of insects that enabled individuals to feed on some previously protected plant group, selection could carry that line into a new adaptive zone. Here it would be free to diversify." Generally, biochemical innovations in insects and plants have been broadly defined, e.g., the ability to feed on a particular plant, in the case of an insect, or the ability to biosynthesize a particular toxin, in the case of a plant. Rarely have specific adaptive traits been identified biochemically or genetically. Considerable insight can be gained by defining with greater precision components of those broad traits that contribute to diversification. Molecular approaches now allow chemical ecologists to characterize those biochemical innovations that are postulated to lead to adaptation and diversification in plant/insect interactions. A case in point involves plants that contain furanocoumarins and the insects that eat them; for both insects and plants, P-450s are essential mediators of the interaction.

Furanocoumarins owe their toxicity to their ability to bind covalently to DNAs and proteins in the presence of ultraviolet light; moreover, in the presence of molecular oxygen they can act as prooxidants that generate toxic oxygen species such as singlet oxygen and superoxide anion. Thus, furanocoumarins can interfere with cellular processes to cause toxicity in a wide variety of organisms, including insects (Berenbaum, 1991, 1995b). Over 200 furanocoumarins have been described from plants. Plant furanocoumarins occur in two structural configurations (Fig. 1): linear furanocoumarins (with the furan ring attached at the 6,7 positions of the benz-2-pyrone nucleus) can cross-link opposing strands in DNA helices as well as bind to proteins, whereas angular furanocoumarins (with the furan ring attached at the 7,8 positions of the benz-2-pyrone nucleus) bind to proteins but form only monoadducts with DNA. Linear furanocoumarins are known from approximately a dozen families, although they are most diverse and ubitquitous in Rutaceae and Apiaceae. Angular furanocoumarins are more narrowly distributed and are found only in a few genera in three plant families (Fabaceae, Rutaceae, Apiaceae); they are most frequently encountered in two tribes in the Apiaceae (Murray et al., 1982).

The biosynthesis of both linear and angular furanocoumarins begins with L-phenylalanine and involves several cytochrome P-450 monooxygenases (Fig. 1). Three P-450s catalyze in sequence the biosynthesis of bergaptol from

demethylsuberosin (6-prenylated umbelliferone) via (+)-marmesin and psoralen (Stanjek et al., 1999; Matern et al., 1999). Although these P-450s have been characterized biochemically, they have not yet been defined at the molecular level. Furanocoumarin biosynthesis, however, is clearly inducible by multiple forms of environmental stress, including insect damage (Zangerl and Berenbaum, 1998; Berenbaum and Zangerl, 1999); in *Apium graveolens*, furanocoumarin biosynthesis is stimulated by jasmonic acid and its analogs (Stanjek et al., 1997). The pattern of furanocoumarin response to insect damage in *Pastinaca sativa* is different from that elicited by mechanical damage (Zangerl and Berenbaum, 1990), raising the possibility that, as in some other plant–insect interactions, insect-derived oral secretions may contain compounds that influence induction of plant biosynthesis (Frey et al., 2000).

Few insects other than lepidopterans specialize on furanocoumarin-containing plants, and, within the Lepidoptera, specialization on these toxic plants is restricted to a handful of families (Berenbaum, 1983). Cytochrome P-450-mediated detoxification of furanocoumarins is associated with specialization on these plants in the Papilionidae, the swallowtail butterflies. Within the family, furanocoumarin-feeding is restricted to the genus *Papilio* within the subfamily Papilioninae; over 75% of these species are associated with furanocoumarin-containing plant hosts. Within the genus, P-450-mediated metabolism of furanocoumarins corresponds to the frequency of ecological exposure. Constitutive levels of activity (associated with host plants unsupplemented with furanocoumarins) against xanthotoxin, a linear furanocoumarin, are high in *Papilio cresphontes*, a specialist restricted to linear furanocoumarin-containing Rutaceae, the putative ancestral host plant family of the group. Constitutive activity is high as well in *P. polyxenes* and *P. brevicauda*, two specialists associated with furanocoumarin-containing Apiaceae. In contrast, P-450-mediated metabolism of xanthotoxin is undetectable in *P. troilus*, a specialist on host plants in the Lauraceae, all of which lack furanocoumarins; furanocoumarin metabolic activity is also not inducible by supplemental dietary furanocoumarins (Cohen et al., 1992).

The molecular basis of furanocoumarin resistance has been characterized at least in part in *P. polyxenes*. Transcripts of two cDNAs, *CYP6B1v1* and *CYP6B1v2*, accumulate in response to supplemental furanocoumarins (Cohen et al., 1992); that these cDNAs encode furanocoumarin-metabolizing P-450s was demonstrated by baculovirus-mediated expression in two different lepidopteran cell lines (Sf9, Tn5) (Ma et al., 1994) (Table 2). These P-450s metabolize linear furanocoumarins with high levels of activity; angular furanocoumarins such as angelicin and sphondin are metabolized poorly, if at all. Expression of CYP6B1 mRNA is tissue-specific, restricted to midgut and, to a lesser extent, fat body (Petersen et al., 2001) (Table 2). Consistent with its function in metabolizing plant allelochemicals, CYP6B1 is expressed only in larval feeding stages; no activity is detectable in eggs, pupae, or adults, which also lack furanocoumarin-metabolizing capabilities (Harrison et al.,

2001). Transcripts of a second P-450, *CYP6B3* (Hung et al., 1995), accumulate in response to a greater variety of linear and angular furanocoumarins than do *CYP6B1* transcripts, but CYP6B3 expressed in baculovirus-infected lepidopteran cells shows only low levels of furanocoumarin-metabolic activity (J.-C. Chen, unpublished).

Insights into regulation of CYP6B1 come from work with the *CYP6B1v3* gene (Prapaipong et al., 1994), with a coding sequence 98% identical to *CYP6B1v1* cDNA. A *cis*-acting regulatory element is necessary for transcriptional induction of P-450s in *P. polyxenes*. Nucleotides spanning the region from −136 to −119 have been identified as the xanthotoxin-responsive xenobiotic response element (XRE-xan) and are required for basal transcription and xanthotoxin inducibility (Prapaipong, 1995; Prapaipong et al., 1994). This element is conserved throughout the known *Papilio* P-450 genes (Table 5), consistent with a role in furanocoumarin regulation. The furanocoumarin inducibility of P-450 genes in other insects (Table 2), including those that rarely, if ever, encounter furanocoumarins, indicate that a search for this element in promoter sequences may be productive.

Members of the *P. glaucus* species group are exceptional in that they represent the most polyphagous species in an otherwise oligophagous family. All six species can utilize rutaceous plants, even when these plants are not within the normal host range (Scriber, 1988). *P. glaucus* and *P. canadensis* are two closely related generalists that are reported on a wide range of host plant families. Despite their similar appearance, they display consistent genetic, physiological, behavioral, and ecological differences (Scriber, 1988; Hagen, 1990; Hagen and Scriber, 1991,

TABLE 5. PUTATIVE XENOBIOTIC-RESPONSIVE ELEMENTS IN *CYP6B* PROMOTERS[a]

	XRE-xan	XRE-AhR	ARE
CYP6B1v3[b]	**ACAATGAC**TGGCAA**AT**TTT**TT**TT	**C**T**C**G**CAAGCA** (−57/−48)	**ATGAC**T**A**TTC (−99/−90)
CYP6B3v1[b]	**A**TA**A**T**TA**TTGATG**A**AT[e]TAT**TT**	**CTCGCAAGCA** (−59/−50)	**ATGAC**TGGCA (−137/−128)
CYP6B4v2[c]	**ACAATGAC**AACAT**AT**AAT**TT**AC	**C**A**C**G**CAAGCA** (−191/−182)	**ATGAC**AACAT (−489/−480)
CYP6B21[c]	**ACAATGAC**AAGAT**AT**AAT**TT**AC	**C**A**C**C**CAAGCA** (−192/−183)	**ATGAC**AAGAT (−437/−428)
CYP6B11[d]	**ACAATGAC**AACAT**AT**AAT**TT**AC	**C**A**C**C**CAAGCA** (−191/−182)	**ATGAC**AA**C**AT (−491/−482)
CYP6B18[d]	**ACAATGAC**AACAT**AT**AAT**TT**AC	**C**A**C**T**CAAGCA** (−191/−182)	**ATGAC**AA**C**AT (−495/−486)

[a] Sequences from Genbank.
[b] *P. polyxenes.*
[c] *P. glaucus.*
[d] *P. canadensis.*
[e] 6 bp missing: TATAAAA.

1995). Geographical separation into northern *P. canadensis* and southern *P. glaucus* has led to different patterns of host plant use due to differences in the frequency of encounter. *P. glaucus* uses hoptree (*Ptelea trifoliata*), a rutaceous plant containing linear furanocoumarins (Murray et al., 1982), whereas *P. canadensis* rarely, if ever, encounters this host, although it can survive on its foliage in the laboratory (Scriber, 1988, 1995). No other reported host of either species is reported to contain furanocoumarins.

Furanocoumarin metabolism in *P. glaucus* is attributable, at least in part, to xanthotoxin-inducible *CYP6B4v1* cDNA and a closely related *CYP6B4v2* genomic DNA that differ in 7/500 amino acids (Hung et al., 1996, 1997). Baculovirus-mediated expression of CYP6B4v1 demonstrated that it metabolizes linear furanocoumarins at high rates (isopimpinellin, bergapten, xanthotoxin) and angular furanocoumarins at lower rates (angelicin, sphondin) (Hung et al., 1997) (Table 2). The metabolic capabilities of this P-450 are, in general, consistent with the frequency and distribution of furanocoumarins present in foliage of *P. trifoliata*, although differences between the overall metabolic profiles of caterpillars and the substrate profiles of CYP6B4v1 *in vitro* suggest that other P-450s may contribute to furanocoumarin metabolism. W. Li et al. (2001) succeeded in identifying two additional cDNAs (*CYP6B23* and *CYP6B24*) and five additional *CYP6B* gene sequences containing full-length or nearly full-length P-450 coding sequences and 5′ promoter sequences ranging from 400 bp to 1.7 kb (*CYP6B12*, *CYP6B16*, *CYP6B17*, *CYP6B20*, and *CYP6B21*) from *P. glaucus*. All of these P-450s are xanthotoxin-inducible (Table 2), but the extent to which they participate in furanocoumarin metabolism is unknown.

Although it rarely encounters furanocoumarins, *P. canadensis* can metabolize certain furanocoumarins at low levels (W. Li et al., 2001). In interspecific comparisons, metabolism rates for both linear and angular furanocoumarins are invariably higher in induced *P. glaucus* larvae than in induced *P. canadensis* larvae. From *P. canadensis*, W. Li et al. (2001) characterized two full-length cDNA sequences (*CYP6B25* and *CYP6B26*) and five gene sequences containing full-length or nearly full-length P-450 coding sequences (*CYP6B11*, *CYP6B13*, *CYP6B14*, *CYP6B15*, and *CYP6B18*), as well as the prematurely truncated open reading frames of *CYP6B19* and *CYP6B22*. As in *P. glaucus*, all of the CYP6B P-450s characterized in *P. canadensis* are xanthotoxin-inducible.

That this ability of xanthotoxin to cause accumulation of furanocoumarin-metabolizing P-450s is maintained in *P. canadensis* even though this species rarely, if ever, encounters furanocoumarins in its host plants suggests the furanocoumarin response cascade might be similar to that found in *P. glaucus*. Consistent with this suggestion is the high degree of similarity in promoter sequences in *CYP6B* genes in these two species. The proximal 5′ flanking sequence (translation start codon to −615) is highly conserved, with 91–98% identity, although the distal region is more variable. The xenobiotic-responsive elements to arylhydrocarbons

(XRE-AhR) and xanthotoxin (XRE-xan) (Prapaipong et al., 1994; Hung et al., 1996), are conserved among all these *CYP6B* genes (except for one position in the XRE-AhR sequence) (W. Li et al., in preparation) (Table 5).

Alignment of the deduced amino acid sequences of the 14 full-length or nearly full-length CYP6B subfamily members in *P. glaucus* and *P. canadensis* reveals that they share an extremely high degree of sequence identity, ranging from 92.8% to 99.8%. Comparison of the deduced protein sequences indicates that variations among these full-length sequences are confined to 64 out of 500 positions. There is, however, conservation of certain critical amino acids. In SRS1, one of the substrate recognition sites that define catalytic activities in P450s (Gotoh, 1992), Phe116 and His117 have been demonstrated to affect the integrity of the catalytic pocket and, thus, to influence the range of furanocoumarins metabolized (Chen et al. 2002). Both residues are conserved in the CYP6B proteins of *P. glaucus* and *P. canadensis*. However, the presence of at least eight differences within four of the substrate recognition sites determining P-450 catalytic properties (Gotoh, 1992; Straub et al., 1994; von Wachenfeldt and Johnson, 1995; Negishi et al., 1996; Roussel et al., 2000) suggests that some of these enzymes may differ from CYP6B1 in their range of furanocoumarin substrates or may even metabolize substrates other than furanocoumarin. This is not an unlikely scenario, given the broad host plant range of these two species. *In vitro*, however, CYP6B4 failed to metabolize representative alkaloids, coumarins, flavonoids, terpenoids, or phenylpropanoids that are naturally encountered within the host plant range (C. Hung, unpublished results).

A comparison of *P. glaucus* and *P. canadensis CYP6B* genes suggests that *P. canadensis* and *P. glaucus* are recently derived from a common ancestor that ostensibly possessed P-450 genes encoding furanocoumarin detoxification enzymes (Berenbaum et al., 1996). Conserved features in the promoter and coding regions of these genes, including the presence of insertions and deletions (W. Li et al., in preparation), most likely occurred prior to or concomitant with divergence of these species; unique features, including differences in substrate recognition sites and variability in promoter sequence upstream of a 640-bp conserved region, may have resulted from relaxed selection in the absence of furanocoumarin-containing hosts and may contribute to lower levels of induction as well as lower rates of metabolism of furanocoumarins in *P. canadensis*.

The isolation of a multitude of *CYP6B* genes from both species indicates that the genomes of both contain multiple closely related genes that are continuously differentiating. Analysis of the promoter and intron structure (W. Li et al., in preparation) indicates that these genes likely arose through repeated duplication of a single progenitor gene, followed by the insertion/deletion of sequences at several sites within the 5′ flanking and intron regions. The fact that *P. canadensis* maintains the ability to induce *CYP6B* genes even though it rarely if ever encounters furanocoumarins suggests that there may still be gene flow between these species in hybrid zones (Hagen, 1990; Scriber et al., 1991). Alternatively, if, in fact, these

species diverged long ago, the possibility exists that the mutation rate is low in regulatory elements or that elimination of P-450 genes from the genome is a slower process than expected in the absence of selection (Kimura et al., 1989; Gonzalez and Gelboin, 1989; Nebert, 1997); as well, the regulatory apparatus may be maintained for responses to other toxins ingested by these two species.

The patterns of evolutionary change in P-450s in *P. glaucus* and *P. canadensis* are paralleled in another pair of closely related polyphagous lepidopterans. *Helicoverpa zea* is a polyphagous noctuid that, despite its broad host range, rarely encounters furanocoumarins; nevertheless, like *P. glaucus* and *P. canadensis*, *H. zea* maintains furanocoumarin-inducible metabolism. From this insect, Li et al. (2000) cloned and sequenced *CYP6B8*, a full-length cDNA encoding protein with 51–99% identity with other members of the CYP6B subfamily. Like transcripts of *CYP6B1* and *CYP6B3* in *P. polyxenes* and *CYP6B* in *P. glaucus* and *P. canadensis*, transcripts of *CYP6B8* accumulate in response to dietary furanocoumarins. In a genomic study that parallels work in *P. glaucus*, X. Li et al. (2001) found a series of closely related genes. Four *CYP6B* genes exist as two pairs of duplicated paralogs with recent gene conversions at the 5′ end. In a comparison with *H. armigera*, a highly polyphagous congener that can hybridize under laboratory conditions with *H. zea*, three groups of orthologous *CYP6B* genes were found: *HzCYP6B8–HaCYP6B7*, *HzCYP6B27–HaCYP6B6*, and *HzCYP6B9–HaCYP6B2/B10*. The degree of divergence in the first two of these groups is extremely low: 1% (amino acid) and 4% (nucleotide) for *HzCYP6B8–HaCYP6B7* and 2% (amino acid) and 4% (nucleotide) for *HzCYP6B27–HaCYP6B6*. This is similar to the allelic variation for P-450s within species. Heliothine *CYP6B* genes share at least 80% identity in amino acids and coding nucleotides. Despite some divergence in coding sequence, the 5′ exon/intron splicing sequences are absolutely identical, and the 3′ intron/exon splicing sequences are also highly conserved. This high level of conservation throughout the gene suggests relatively recent gene duplication.

Despite the taxonomic distance between the groups, heliothine and papilionine P-450s share several similarities. All *Papilio* P-450 proteins characterized to date are furanocoumarin-inducible, as is CYP6B8 in *Helicoverpa* (Table 2). A striking number of amino acids are conserved in SRS1 and SRS4 in all CYP6B proteins. Among those absolutely conserved are Phe 116 and His 117, required for furanocoumarin metabolism in CYP6B1 (Chen et al., 2002). *P. glaucus* B4/B5 share almost identical nucleotide (coding) regions and amino acid sequences (Hung et al., 1996), as do *H. zea* paralogs *CYP6B8* and *CYP6B28*. The presence of multiple P-450s in two still-diverging pairs of polyphagous species raises the possibility that polyphagy may arise in specialized groups via extensive gene duplication. Such gene duplication could allow for rapid acquisition of new substrates; indeed, rapid gene duplication is thought to underlie the tremendous diversity of P-450s in the genome of the human (Nebert, 1997), arguably the most polyphagous species on the planet.

The degree to which polyphagy depends on a predisposition to gene duplication may be lineage-dependent. In Lepidoptera, for example, the vast majority of species (including the putative ancestors of the two pairs of polyphagous species examined here) are relatively oligophagous. In a lineage with relatively substrate-specific P-450s, gene duplication with subsequent acquistion of new function may be a mechanism for shifting from oligophagy to polyphagy. However, polyphagy may also reflect physiological adaptation via P-450s with broad substrate specificity; such appears to be the case, for example, in humans, in which individual P-450s can turn over dozens of biosynthetically and structurally unrelated substrates (Berenbaum, 1999). Behavioral adaptations are also a factor in the acquistion of the polyphagous habit. Again, in humans, a spectacularly broad diet may minimize the exposure of an individual to any particular group of dietary toxins. Whether there is any relationship at all between P-450 diversity and diet breadth, however, cannot be determined definitively until the P-450 inventories of a larger number of species have been determined.

In contrast with the evolution of polyphagy, the presence of highly conserved promoter elements that play a role in furanocoumarin-responsiveness as well as highly conserved features of substrate recognition sites in three species within the genus *Papilio* suggest that strong selection for specialization may manifest itself in the genome. Indeed, extensive investigation of *P. polyxenes*, a specialist on furanocoumarin-containing plants that experiences a much narrower and more predictable array of allelochemicals in its host plants, has yet to reveal extensive gene diversity. The key innovation in allowing species in the genus *Papilio* to utilize and diversify on furanocoumarin-containing plants may well have been the linkage between a promoter element allowing responsiveness and a coding sequence allowing rapid and efficient metabolism (Berenbaum et al., 1996; Stevens et al., 2000).

CONCLUSIONS

The field of chemical ecology, the study of the ways in which chemicals affect the distribution, abundance, and interaction of organisms, really did not gain a distinct identity until 1970, when a book appeared, titled *Chemical Ecology* (Sondheimer and Simeone, 1970). Shortly thereafter, in 1974, the *Journal of Chemical Ecology* was founded, "devoted to promoting an understanding of the origin, function, and significance of natural chemicals that mediate interactions within and between organisms." Following the first Gordon Conference organized around the subject of plant–herbivore interactions in 1980, the International Society for Chemical Ecology was organized. Today, membership in the society includes not only chemists and ecologists but a wide range of life scientists. As practiced today, the field of chemical ecology involves not only the fields of natural products chemistry

and ecology but also such subdisciplines of biology as behavior, evolution, genetics, and physiology.

In many subdisciplines within biology, the use of molecular tools and approaches has led to a tremendous explosion of productivity. These tools and approaches hold considerable promise for the field of chemical ecology. In particular, the possibility now exists to identify mechanisms underlying familiar and well-characterized ecological and behavioral phenomena—host plant specificity, mate choice, territoriality, allomonal defense, and the like. The enormous advantage of elucidating mechanisms is that it allows for a more complete understanding of complex phenomena as well as more refined ability to make predictions about newly observed phenomena. At the same time, the field of chemical ecology has much to offer to genome scientists; only those intimately familiar with the small molecules that affect ecology and behavior can begin to hazard a guess as to the function of the macromolecules that provide the blueprints for behavior and ecology. Chemical ecology has, since its inception, been an inherently multidisciplinary field. Traditionally, it has been a synthesis of a physical science (chemistry) and a biological science (ecology). At the beginning of the new century, it seems particularly apt to expand the multidisciplinary nature of the field to take advantage of ways to think about and new tools to tackle familiar research challenges.

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THE ROLE OF MONOTERPENES IN RESISTANCE OF DOUGLAS FIR TO WESTERN SPRUCE BUDWORM DEFOLIATION

ZHONG CHEN,[1] THOMAS E. KOLB,[1,*] and KAREN M. CLANCY[2]

[1]*School of Forestry*
Northern Arizona University
Flagstaff, Arizona 86011-5018

[2]*Rocky Mountain Research Station*
2500 S. Pine Knoll Drive
Flagstaff, Arizona 86001-6381

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Abstract—We conducted defoliation experiments with 7- to 8-year-old clones of Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco var. *glauca*] to assess the role of monoterpenes as a resistance mechanism to western spruce budworm (*Choristoneura occidentalis* Freeman) defoliation. The grafted clones were derived from mature trees that showed resistance or susceptibility to budworm defoliation in the forest. All clones were exposed to either budworm defoliation or nondefoliation treatments in 1998 and 1999 under greenhouse conditions. We found that the total concentration of monoterpenes in current-year foliage varied greatly between two consecutive years in clones in the greenhouse and in their corresponding mature trees in the forest. Fractional composition of different monoterpenes was similar between different years and between clones and mature trees, indicating genetic control of this trait. Two different defoliation experiments were conducted to assess the importance of budburst phenology as a factor determining host plant resistance. In the 1998 experiment, budworm feeding was matched to the budburst of each individual plant. Monoterpene concentration was high in 1998, and budworm potential fitness was greater on clones from the resistant mature trees that had lower concentrations of total monoterpenes. In the 1999 experiment, budworm feeding was matched to budburst of the whole population of plants in order to mimic conditions similar to insects feeding on trees in the field. The concentration of monoterpenes was low in 1999, and budworm fitness was not related to monoterpenes. Total monoterpene concentration was negatively related to foliar nitrogen concentration, suggesting that C/N balance may affect monoterpene synthesis in needles. However, tree growth was not related to total monoterpene concentration. We concluded that

* To whom correspondence should be addressed. E-mail: Tom_Kolb@nau.edu

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expression of differences in budworm resistance among Douglas fir genotypes might be caused by interactions among multiple resistance mechanisms such as needle monoterpenes and tree budburst phenology.

Key Words—Budburst phenology, *Choristoneura occidentalis*, herbivory, monoterpenes, plant-insect interactions, *Pseudotsuga menziesii*.

INTRODUCTION

Terpenes are the largest and most diverse group of plant secondary metabolites. At least 15,000 terpenoids have been described and perhaps thousands more are yet to be discovered (Gershenzon and Croteau, 1991). Monoterpenes (i.e., C_{10} hydrocarbons) are one of the major terpene groups and are widespread in plants, functioning as either deterrents or attractants to herbivores (Hedin et al., 1974; Kogan, 1975). They are also involved in numerous physiological and ecological interactions in forest ecosystems (Harborne, 1991; Gershenzon, 1994; Langenheim, 1994; Lerdau et al., 1995). Monoterpene concentration and composition in coniferous trees are influenced by tree genotype (von Rudloff and Rehfeldt, 1980; Gershenzon and Croteau, 1991; Hanover, 1992; Gershenzon, 1994) and environmental factors such as availability of nitrogen (Lerdau et al., 1995; McKinnon et al., 1998), water, and sunlight (Johnson et al., 1997). Further, monoterpene concentration and composition can change during tissue maturation (Wagner et al., 1989; Gambliel and Cates, 1995; Zou and Cates, 1995) and can vary among different tissues within the same tree (Latta et al., 2000).

The association between terpenes and resistance of host trees to insect herbivores has been investigated in several conifers (Annila et al., 1984; McClure and Hare, 1984; Redak and Cates, 1984; Hanula et al., 1985; Wilkinson, 1985; Cates et al., 1987; Cates and Redak, 1988; Cates and Zou, 1990; Clancy, 1991a; Clancy et al., 1992, 1993; Wagner and Zhang, 1993; Felipe et al., 1994; Tomlin et al., 1997; Manninen et al., 1998; Nault et al., 1999). However, the role of terpenes as a resistance mechanism remains controversial. Several studies have concluded that monoterpenes are an important mechanism of resistance to insect herbivores in conifers (McClure and Hare, 1984; Cates et al., 1987; Cates and Zou, 1990; Felipe et al., 1994; Tomlin et al., 1997), whereas other studies have not (Annila et al., 1984; Wilkinson, 1985; Clancy, 1991a, 1993, 2001; Clancy et al., 1992, 1993; Wagner and Zhang, 1993; Manninen et al., 1998; Nault et al., 1999). This controversy likely arises from the many influences on conifer monoterpene concentration and composition (e.g., tree genotype, ontogeny, environment, and genotype–environment interaction), the evolution of different resistance mechanisms for different host plant–insect systems, and the possible involvement of several mechanisms in host plant resistance.

Evidence for an important role of monoterpenes as a tree resistance mechanism is also mixed for the subjects of our study, Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco var. *glauca*] and western spruce budworm (*Choristoneura occidentalis* Freeman). Some monoterpenes, such as tricyclene, camphene, and bornyl acetate, have been reported to adversely affect western spruce budworm performance on Douglas fir (Cates et al., 1983; Redak and Cates, 1984; Cates and Zou, 1990; Zou and Cates, 1995). In contrast, other studies have found no difference in these monoterpene components between mature Douglas fir trees that showed resistance versus susceptibility to budworm defoliation in the forest (Clancy, 1991a, 2001; Clancy et al., 1993). Further, Clancy et al. (1992) and Clancy (1993) reported little influence of monoterpenes that were microencapsulated in an artificial diet on budworm performance in a three-generation bioassay unless components such as camphene, bornyl acetate, β-citronellol, and linalool were at extremely high concentrations that rarely occurred in forest trees.

The overall objective of this study is to further understand the role of monoterpenes as a resistance mechanism of Douglas fir to budworm defoliation. We used data on total monoterpene concentration and composition from resistant and susceptible mature trees and clones of these trees in the greenhouse to address these questions: (1) Does foliar monoterpene concentration and composition differ between tree genotypes that were resistant to budworm defoliation versus genotypes that were susceptible? (2) Does variation in monoterpene concentration affect budworm performance in a pattern consistent with field observations of tree resistance? (3) Is the influence of foliar monoterpenes on budworm performance mediated by the degree of synchrony between budworm feeding and tree budburst phenology? (4) Does budworm defoliation induce changes in monoterpene concentration and composition? (5) Does carbon allocation to monoterpene synthesis compromise allocation to tree radial growth?

METHODS AND MATERIALS

Douglas Fir Trees and Clones. Our experimental plant material consisted of clones derived from mature Douglas fir trees that differed in western spruce budworm defoliation under field conditions (Clancy et al., 1993). The mature Douglas fir trees were from sites on the Pike National Forest near Deckers, Colorado (105.23°W, 39.23°N, elevation 2573 m) and the Kaibab National Forest near Jacob Lake, Arizona (112.22°W, 36.71°N, elevation 2774 m). At the time the trees were identified (1988 and 1989), most of the trees at the sites had sustained moderate to severe budworm defoliation for at least several years, as determined from their growth form and general condition. We selected seven phenotypically resistant trees at the Pike National Forest site and five phenotypically resistant trees

at the Kaibab National Forest site by identifying trees with full crowns and little other evidence of budworm damage. These trees were visually distinct from other trees in the stand that were characterized as phenotypically susceptible based on their defoliated crowns. Each resistant tree was paired with a nearby (within 30 m) susceptible tree of similar size (height and dbh) and microsite (slope and aspect). In other words, the pairs of resistant and susceptible trees were matched as closely as possible to minimize any size-, age-, or microsite-related effects that could confound effects associated with different levels of herbivory. We deliberately chose pairs at each site that represented a range of size (i.e., age) classes. The age of the 24 trees ranged between 45 and 123 years [79.3 $\pm$ 4.1 years (mean $\pm$ SE)]; height ranged between 6.4 and 14.9 m (10.4 $\pm$ 0.5 m); dbh ranged between 15 and 40 cm (25.3 $\pm$ 1.3 cm). We measured radial growth increment on these trees over each 5-year interval between 1966 and 1990 on increment cores sampled at breast height (one sample per tree) with a binocular microscope equipped with an ocular micrometer (Clancy et al., 1993).

We cloned each of the 24 mature trees by whip-grafting branches collected from the lower third of the crown onto 1-year seedling rootstocks in 1991 and 1992. This is a common and widespread technique for reproducing mature tree characteristics in a smaller plant (Hartmann and Kester, 1983; Zobel and Talbert, 1984). Such cloning resulted in the fixation of the genotype and tissue developmental stage of mature trees but not tree environment. All cloned trees were grown in plastic pots (15-liter volume) containing a mixture of screened peat moss and vermiculite in the greenhouse; they were regularly watered and fertilized during the growing season. Cloned trees averaged 2.11 $\pm$ 0.03 cm (mean $\pm$ 1 SE, here and throughout) in base stem diameter (approximately 1 cm above the graft), 93.5 $\pm$ 1.6 cm in height, and 45.9 $\pm$ 0.7 cm in crown diameter (averaged over two directions) in spring 1998 before the start of the budworm defoliation experiments.

Experimental Design and Budworm Defoliation. The experiment had a completely randomized block design composed of six blocks, each containing 48 clonally propagated trees [i.e., 2 treatments (budworm defoliation versus control) $\times$ 2 traits (resistant versus susceptible)/pair $\times$ 12 pairs]. In total, 288 cloned trees were included in the experiments. However, 11 trees died before the experiment started; therefore, there were actually four to six replications of each treatment combination for each of the 12 pairs. In order to test the role of budburst phenology as an influence on budworm performance, we conducted the budworm defoliation experiment differently in 1998 and 1999. The budworm larvae used in our study were from our laboratory cultures of diapausing and nondiapausing western spruce budworms, maintained in the Entomology Laboratory at Rocky Mountain Research Station, Flagstaff, Arizona. The nondiapausing colony has growth rates and feeding behavior similar to a wild population (Leyva et al., 1995).

In the 1998 experiment, nondiapausing third- and primary fourth-instar budworm larvae (one larva to five terminal buds) were used to defoliate each cloned

tree when approximately 50% of its buds were in the fourth (i.e., columnar) budburst development stage. This stage is highly suitable for budworm feeding (Shepherd, 1983). Because budworm larval feeding was purposely matched to the fourth budburst stage of each clone, the effect of genetic variation in budburst phenology among trees on budworm feeding was minimized. In the 1999 experiment, diapausing second-instar larvae in hibernacula (one larva to four terminal buds) were introduced to the same clones that were defoliated by budworms in 1998. However, larvae were placed on all trees at the same date when approximately 50% of all terminal buds in the population were in the second (i.e., yellow) budburst stage (Shepherd, 1983). This schedule of larval introduction allowed genetic differences in budburst phenology among trees to influence the developmental stage of buds available for budworm feeding, as can occur in Douglas fir forests (Clancy et al., 1993). Our measurements of budburst phenology of nondefoliated clones in the greenhouse in 1998 revealed that clones of susceptible trees broke bud earlier than clones of resistant trees (Chen et al., 2001a), and the same pattern occurred for the mature trees in the forest (Clancy et al., 1993).

Other experimental procedures were the same in both years. Both defoliated and nondefoliated tree clones were caged with nylon "No-See-um" netting bags (The Rain Shed Corp., Corvallis, Oregon) that allowed approximately 80% of full light to penetrate in order to contain larvae and create similar growing conditions for all clones. The bags were not removed until 95% of the larvae pupated, which required approximately five weeks.

Budworm Bioassay. Most of the pupae were removed from defoliated tree clones within 24–48 hours of pupation. Male and female pupae were separately weighed (to the nearest 0.1 mg), and sorted into trays based on clone genotype. The pupae were refrigerated at 10°C for up to seven days until an adequate number of male and female pupae were collected from the same clone genotype for mating, which occurred in brown paper mating bags at room temperature (20°C). Female pupal weight was used to estimate the number of oocytes per female or potential fecundity (Wagner et al., 1987).

When about 10% of the moths had emerged in the mating bags, freshly clipped Douglas fir foliage was added for adult oviposition substrate. Once the foliage was added, the moths were allowed to mate and oviposit for 7–10 days. Next, the foliage was removed, and the number of next-generation (noted as F_1) egg masses larger than 4 mm was counted. Then, mating bags were frozen to kill the adult moths, and the unemerged pupae (i.e., dead) in each bag were counted and sexed to determine percent survival through the pupal stage and the number of female moths that had emerged. The collected F_1 egg masses were placed in Petri dishes, sealed inside plastic bags, and incubated in the laboratory (20°C) for 7–10 days to determine if the egg masses were viable (i.e., if $\geq$50% of the eggs in the mass hatched).

The measurement of budworm potential fitness was based on the following population growth model modified from Clancy (1991b):

$$\begin{aligned}\text{Number of } F_1 \text{ larvae } (N) = {} & [\text{number of parent generation (noted as } P_1) \text{ larvae}] \\ & * (P_1 \text{ proportion cohort survival to the adult stage}) \\ & * (P_1 \text{ average potential fecundity per female}) \\ & * (F_1 \text{ proportion of egg masses with viable eggs})\end{aligned}$$

This model estimated the number of first instars alive at the beginning of the F_1 generation, assuming that clones from the resistant and susceptible trees had equal population sizes at the beginning of the P_1 generation (i.e., we assumed that all populations started with a single P_1 larva). Cohort survival to the adult stage was based on survival through the larval and pupal stage (multiplied together) when we used data from the 1998 experiment. For the 1999 experiment, this value was based on survival through the pupal stage alone because the data on larval survival were unavailable due to the survival of some residual F_1 larvae on some trees from the 1998 experiment.

We acknowledge that our estimations of budworm potential fitness were inflated in 1998 (because of the lack of data on survival of first-instar larvae to the third or fourth instars) and in 1999 (because of the lack of reliable data on survival of second-instar larvae to the pupal stage). However, the lack of these data did not influence our comparisons between the potential fitness of budworms after feeding on clones of resistant and susceptible trees within each experimental year (i.e., the biases were consistent between the resistant and susceptible trees in each year).

Foliage Collection and Terpene Analysis. Current-year foliage was sampled from mature trees in late June of the 1989 and 1990 growing seasons, corresponding to the late-instar feeding period of the western spruce budworm (foliage was at the feather duster, or seventh developmental stage) (Clancy et al., 1993). One branch was clipped at random from the mid-crown area of the north, south, east, and west quadrants of each tree; the needles collected from these quadrants were pooled for each tree prior to chemical analysis. Although we have previously reported the foliar monoterpene data for the mature trees (Clancy et al., 1993; Clancy, 2001), this study is the first comparison of foliar monoterpenes in the parent trees and their corresponding clones.

Current-year foliage from the clonal trees in the greenhouse was sampled in 1998 and 1999 when late instars of the budworm were actively feeding on the defoliated trees, and the foliage was at the seventh (or feather duster) developmental stage (Shepherd, 1983). Two clusters of current-year foliage were clipped at random from the upper third of the crown. The foliage sampled from defoliated trees was not directly damaged by budworms. The foliage sampled in each year was pooled over the four to six trees from the same clone and treatment (i.e., budworm defoliation versus control). Therefore, no block effect was included in

the analysis of terpene data. A total of 48 pooled foliage samples were used for chemical analysis [i.e., 2 treatments (defoliated versus control) $\times$ 2 traits (resistant versus susceptible clones) $\times$ 12 pairs].

Foliage samples were sealed in plastic bags and temporarily stored in a freezer before being transferred to an ultralow freezer for long-term storage (up to seven months) at -60°C before being analyzed. Approximately 250 mg of needle tissue was extracted from each sample in 5 ml pentane. The extracts were analyzed with a Hewlett Packard 5890 series II gas chromatograph containing an HP-1 capillary column (30 m $\times$ 0.25 mm). Monoterpene concentrations were calculated on a fresh mass basis (Clancy et al., 1993) because foliage samples collected from the clonal trees were at the same phenological stage and were grown under similar environments; thus, they had little variation in moisture content. All chemical analyses were conducted by the Analytical Services Laboratory at Northern Arizona University.

Statistical Analysis. Since the sampled Douglas fir population from the Pike National Forest site (Colorado) was not genetically differentiated from the sampled population from the Kaibab National Forest site (Arizona) based on an isoenzyme study (Chen et al., 2001b), we treated the 12 pairs of trees from these two sites as one population for statistical analysis. We used a multivariate analysis of variance (MANOVA) to investigate the overall effect of tree pair (i.e., 12 pairs of resistant and susceptible mature trees from which clones were derived), trait (resistant versus susceptible), treatment (defoliation versus non-defoliation), and their interactions on variations in total monoterpene concentration and fractional composition (i.e., percentage) of different monoterpenes. After that, we performed a univariate analysis of variance (ANOVA) test with the three-way interaction term as the error term for each monoterpene component using the same model as described for the MANOVA. Since we measured multiple response variables (e.g., 11 different terpenes) from the same set of experimental units, we used a Bonferroni correction to the P value we accepted as indicating significant differences in the univariate ANOVAs in order to maintain the correct P value of 0.05 for the type I error rate over the whole set of univariate ANOVAs. For this study, we focused on the effects of trait, defoliation treatment, and their interaction because they were most relevant to our research questions. Spearman's rank correlation coefficient (SAS Institute, 1995) was used to compare the rank order of percent composition of different monoterpene components between years and between clones and mature trees.

Finally, we used linear regression to examine the association between: monoterpenes and budworm potential fitness in the 1998 and 1999 greenhouse experiments with clones; the most recent 5-year radial growth (1986–1990) of mature trees in the forest and total monoterpene concentration in 1989 and 1990; and total monoterpene concentration of clones and their corresponding mature trees.

RESULTS

Monoterpene Composition and Structure. Eleven monoterpenes were detected in the needles of cloned trees in greenhouse experiments. The rank order of monoterpene concentration in 1998 was bornyl acetate, camphene, limonene, β-pinene, α-pinene, unknown A, unknown C, myrcene, unknown B, tricyclene, and terpinolene (Table 1). Five monoterpenes, α-pinene, camphene, β-pinene, limonene, and bornyl acetate, accounted for approximately 78% of total monoterpenes in 1998 and 86% in 1999 (Table 1). Fractions of bornyl acetate and limonene increased from 1998 to 1999 ($P \leq 0.017$), fractions of camphene, β-pinene, and α-pinene did not change ($P \geq 0.239$), and unknown monoterpenes A, B, C, and terpinolene declined ($P \leq 0.002$) (Table 1). Based on Spearman's rank correlation coefficient, the rank order in concentration of monoterpenes in 1998 and 1999 was highly correlated ($\rho = 0.955$, $P < 0.001$), indicating high consistency of fractional composition over years.

The same 11 monoterpenes present in clones were also present in mature tree foliage (Table 1). The rank order of fractional composition of monoterpenes in mature trees was consistent between 1989 and 1990 (Spearman's $\rho = 0.734$, $P = 0.010$). Moreover, monoterpene fractional composition was similar between mature trees and clones ($\rho = 0.682$–0.902, $P = 0.021$–0.001, depending on years).

Total monoterpene concentration of clones was greater in 1998 than 1999 by approximately 245% ($P < 0.001$), whereas the total monoterpene concentration in mature trees was approximately 315% greater in 1990 than in 1989 ($P < 0.001$) (Table 1). Further, variation in total monoterpene concentration among individual ramets ($r = 0.164$, $P = 0.269$, $N = 48$) and mature trees ($r = 0.339$, $P = 0.104$, $N = 24$) was not significantly correlated between consecutive sampling years. Moreover, there was no clear relationship in total monoterpene concentration between mature trees and clones. For example, total foliar monoterpene concentrations were positively, but weakly, correlated between mature trees in 1989 and clones in both 1998 and 1999 ($P \leq 0.067$; Figure 1). However, this was not the case for mature trees in 1990 ($P > 0.338$) (data not shown).

Effect of Tree Resistance Trait and Treatment on Monoterpenes. Monoterpene concentration and fractional composition in foliage of clones differed among levels of the experimental factors in the 1998 and 1999 greenhouse experiments (MANOVA, $P < 0.001$) (Table 2). Tree pair and the interaction between pair and trait (resistant versus susceptible) were significant (MANOVA, $P \leq 0.002$) in both years, indicating that both concentration and fraction of monoterpenes differed among the clones from 12 pairs of trees and that the difference between the resistant and susceptible trees within each pair was not consistent.

In the 1998 experiment, trait (resistant versus susceptible) had a marginally significant overall effect on monoterpene concentrations (MANOVA, $P = 0.059$) (Table 2). Further univariate ANOVA for each monoterpene component

TABLE 1. COMPARISON OF MONOTERPENE FRACTIONAL COMPOSITION (% OF TOTAL MONOTERPENE CONCENTRATION) AND TOTAL MONOTERPENE CONCENTRATION IN CURRENT-YEAR FOLIAGE FROM DOUGLAS FIR CLONES AND MATURE TREES OVER TWO CONSECUTIVE SAMPLING YEARS[a]

Monoterpene (abbreviation)	Clones ($N = 48$)					Mature trees ($N = 24$)				
	1998		1999			1989		1990		
	Mean	SE	Mean	SE	P^*	Mean	SE	Mean	SE	P
Bornyl acetate (bc), %	17.68	0.81	20.73	0.96	0.017	27.14	1.67	14.62	0.96	0.001
Camphene (ca), %	16.75	0.73	17.81	0.76	0.239	19.32	1.22	15.98	0.96	0.037
Limonene (lm), %	15.49	0.81	19.62	1.09	0.003	12.71	1.14	11.7	0.51	0.422
β-Pinene (bp), %	15.01	0.94	15.54	1.07	0.712	16.68	1.44	11.06	0.91	0.002
α-Pinene (ap), %	12.88	0.36	12.66	0.37	0.672	13.56	0.58	12.21	0.59	0.110
Unknown A (ua), %	8.09	0.81	2.75	0.49	0.001	3.74	0.77	18.11	1.73	0.001
Unknown C (uc), %	4.51	0.54	2.42	0.37	0.002	1.12	0.43	7.35	1.05	0.001
Myrcene (my), %	3.63	0.19	4.29	0.28	0.051	1.69	0.25	2.67	0.13	0.001
Unknown B (ub), %	3.01	0.24	1.58	0.25	0.001	0.45	0.06	3.09	0.32	0.001
Tricyclene (tr), %	2.21	0.11	2.03	0.09	0.234	3.14	0.29	2.3	0.13	0.012
Terpinolene (tp), %	0.92	0.06	0.57	0.09	0.002	0.45	0.06	0.91	0.05	0.001
Total concentration, ppm	1489.69	105.83	432.00	34.46	0.001	622.08	83.99	2579.33	196.46	0.001

[a] The P value indicates whether there were differences between two sampling years.

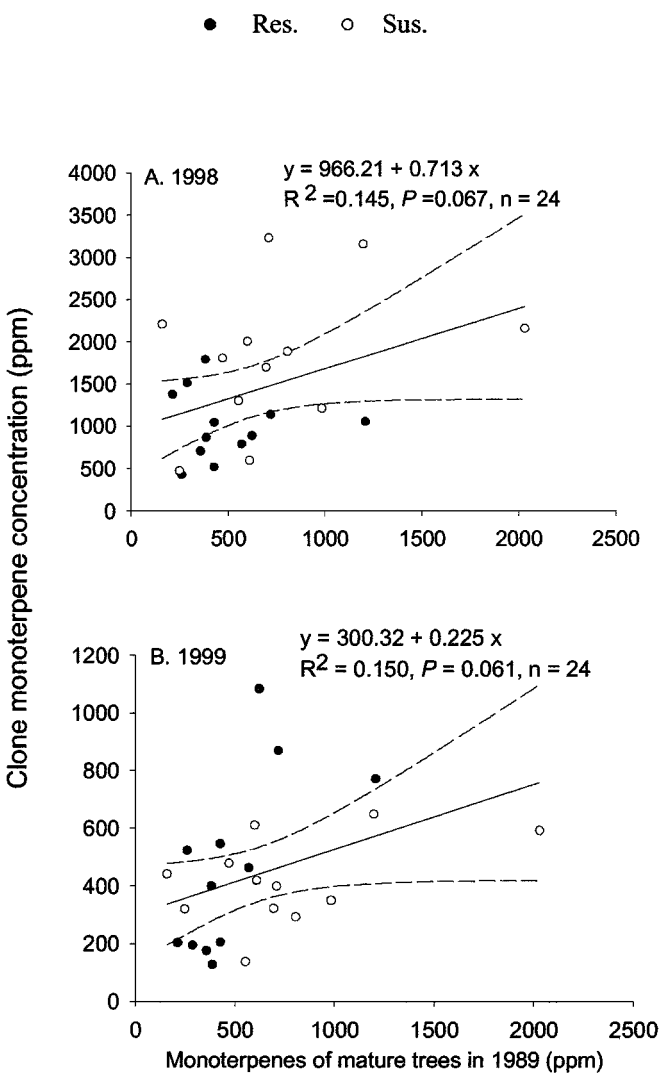


FIG. 1. Regression of foliar concentration (ppm fresh weight) of total monoterpenes in current-year foliage of clones derived from mature Douglas fir trees against concentrations in the original parent trees. All needles were at the seventh (i.e., feather duster) developmental stage when they were sampled. Clonal data is shown for 1998 (A) and 1999 (B) and is plotted against data from the parent trees for 1989. Data are shown for mature trees that appeared to be resistant (●) versus susceptible (○) to western spruce budworm defoliation. Dashed lines around the regression line indicate the 95% confidence limits for the mean response, and the equations for the regression lines and their associated statistics are shown on the graphs.

TABLE 2. RESULTS OF MULTIVARIATE ANALYSIS OF VARIANCE WITH WILKS' LAMBDA TEST FOR EFFECTS OF TREE PAIR, TRAIT, AND TREATMENT ON CONCENTRATION (FRESH-WEIGHT-BASED) AND FRACTIONAL COMPOSITION (% OF TOTAL MONOTERPENE CONCENTRATION) OF 11 MONOTERPENES FROM 1998 AND 1999 GREENHOUSE EXPERIMENTS WITH DOUGLAS FIR CLONES[a]

Source of variation	Wilks' lambda test (Num DF, Den DF)	*P*			
		1998		1999	
		Concentration	Composition	Concentration	Composition
Pair	121, 23	<0.001	<0.001	<0.001	<0.001
Trait	11, 1	0.059	0.233	0.491	0.608
Treatment	11, 1	0.550	0.201	0.518	0.595
Pair × trait	121, 23	<0.001	<0.001	<0.001	<0.001
Pair × treatment	121, 23	0.281	0.294	0.423	0.892
Trait × treatment	11, 1	0.533	0.521	0.219	0.429

[a] *P* value represents the probability for the null hypothesis of no overall effect from each source of variation.

indicated that clones from resistant trees generally had a lower concentration of monoterpenes than clones from susceptible trees (ANOVA, $P \leq 0.021$) (Table 3; Figure 2A). Moreover, clones of resistant trees had a lower average total monoterpene concentration (1181 ± 75 ppm) than clones from susceptible trees in 1998 (1798 ± 75 ppm) ($P < 0.001$; Table 3). In contrast, overall monoterpene fractional composition did not differ between clones from resistant and susceptible trees in 1998 (MANOVA, $P = 0.233$; Table 2). However, univariate ANOVA suggested that clones from resistant trees had a higher fraction of camphene and bornyl acetate and a lower fraction of β-pinene and unknown C than clones from susceptible trees ($P \leq 0.004$; Table 3) (Figure 2C). Budworm defoliation treatment in 1998 did not influence the overall concentration or fractional composition of monoterpenes (MANOVA, $P \geq 0.201$; Table 2). However, the univariate ANOVAs suggested a reduction in the fraction of myrcene and limonene in defoliated versus nondefoliated tree clones ($P \leq 0.005$; Table 3; Figure 3C).

Clones from susceptible trees had a greater ratio of total monoterpene concentration to nitrogen concentration (i.e., monoterpene/N ratio; 0.083 ± 0.005) than clones from resistant trees (0.054 ± 0.005) in the 1998 experiment ($P = 0.001$). Furthermore, budworm defoliation caused a marginal increase in the monoterpene/N ratio (0.075 ± 0.005) compared to the control (0.062 ± 0.005) in 1998 ($P = 0.088$). The interaction between tree trait and treatment did not influence the concentration or fractional composition of monoterpenes in 1998 (MANOVA, $P \leq 0.533$; Table 2) or the monoterpene/N ratio, indicating similar effects of defoliation on clones from resistant and susceptible trees. Univariate ANOVA also supported the absence of any significant trait × treatment interactions ($P \geq 0.033$; Table 3).

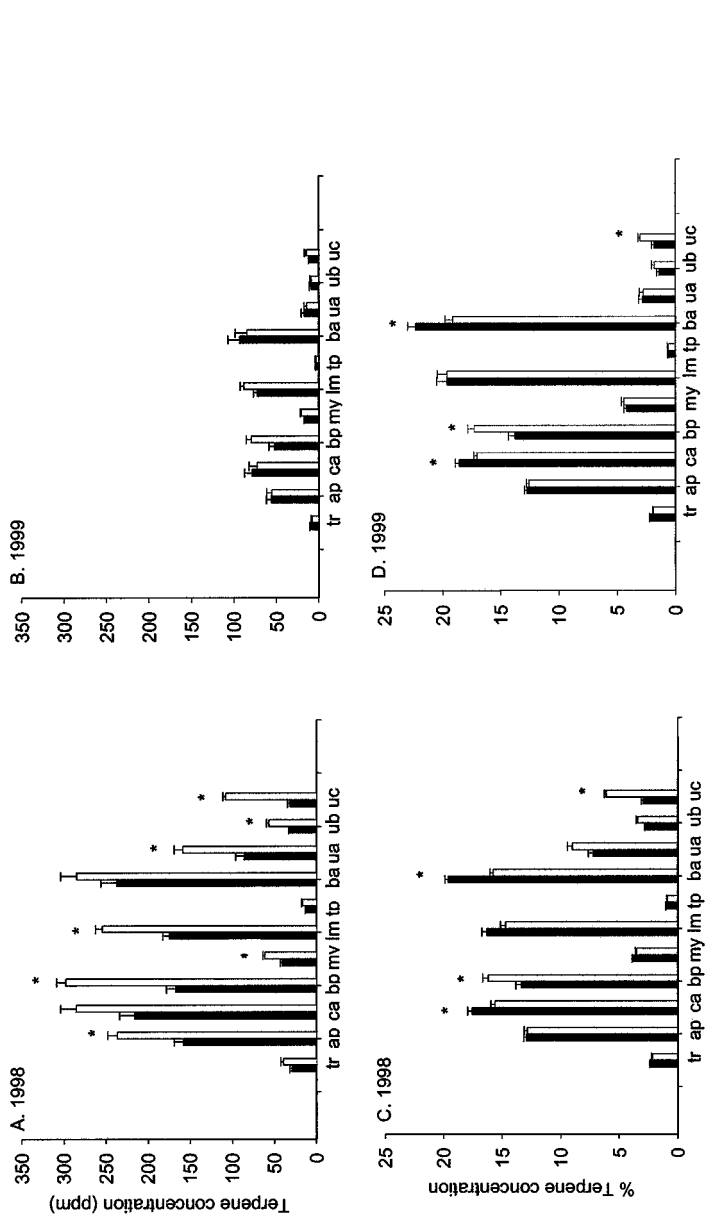


FIG. 2. Mean (+1 SE) concentration (A, B) and fractional composition (C, D) of 11 monoterpene components in Douglas fir needles sampled in the 1998 (A, C) and 1999 (B, D) greenhouse experiments for clones from mature Douglas fir trees that appeared to be resistant (■) versus susceptible (□) to western spruce budworm defoliation. Abbreviations represent 11 monoterpenes defined in Table 1. Asterisks (*) indicate there was a significant difference between the resistant and susceptible clones (ANOVA, Bonferroni adjusted $\alpha \leq 0.005$).

TABLE 3. RESULTS FROM UNIVARIATE ANALYSIS OF VARIANCE TESTS (F TEST AND PROBABILITY VALUES) FOR EFFECTS OF TRAIT, TREATMENT, AND THEIR INTERACTION ON CONCENTRATION (FRESH-WEIGHT-BASED) AND FRACTIONAL COMPOSITION (% OF TOTAL MONOTERPENE CONCENTRATION) OF EACH MONOTERPENE COMPONENT OF DOUGLAS FIR CLONES IN 1998 EXPERIMENT WHEN BUDWORM FEEDING WAS MATCHED TO BUDBURST OF EACH INDIVIDUAL PLANT[a]

	Trait (resistant vs. susceptible)				Treatment (defoliation vs. nondefoliation)				Trait × treatment			
	Fresh wt		%		Fresh wt		%		Fresh wt		%	
1998 data	$F_{1,11}$	P	$F_{1,11}$	P	$F_{1,11}$	P	$F_{1,11}$	P	$F_{1,11}$	P	$F_{1,11}$	P
Tricyclene	7.74	0.018	1.96	0.189	3.66	0.082	3.95	0.072	2.38	0.151	1.72	0.216
α-Pinene	24.34	0.001	0.05	0.821	3.13	0.104	1.36	0.268	2.57	0.137	0.01	0.943
Camphene	7.27	0.021	12.99	0.004	3.91	0.074	7.05	0.022	2.78	0.124	2.13	0.172
β-Pinene	69.84	0.001	13.35	0.004	0.11	0.748	3.06	0.108	0.98	0.343	1.94	0.192
Myrcene	51.38	0.001	3.77	0.078	0.01	0.977	11.96	0.005	2.37	0.152	2.09	0.176
Limonene	51.81	0.001	7.27	0.021	0.01	0.980	13.71	0.004	2.62	0.134	2.54	0.139
Terpinolene	9.31	0.011	2.29	0.158	8.23	0.015	10.17	0.009	5.96	0.033	5.59	0.038
Bornyl acetate	3.06	0.108	94.13	0.001	2.85	0.119	9.61	0.010	3.71	0.081	5.34	0.041
Unknown A	24.73	0.001	8.59	0.014	0.74	0.409	0.25	0.628	0.76	0.403	0.01	0.919
Unknown B	56.88	0.001	10.14	0.009	0.84	0.381	0.12	0.742	0.56	0.472	0.10	0.751
Unknown C	274.74	0.001	210.25	0.001	0.28	0.610	0.18	0.678	0.00	0.980	0.00	0.947
Total	33.65	0.001			2.27	0.161			2.97	0.113		

[a] The Bonferroni adjusted P value of $\alpha = 0.005$ (i.e., 0.05 divided by 11) was used to determine significance, in order to maintain the correct P value of 0.05 for the type I error rate over the whole set of univariate ANOVAs for the 11 individual terpene compounds.

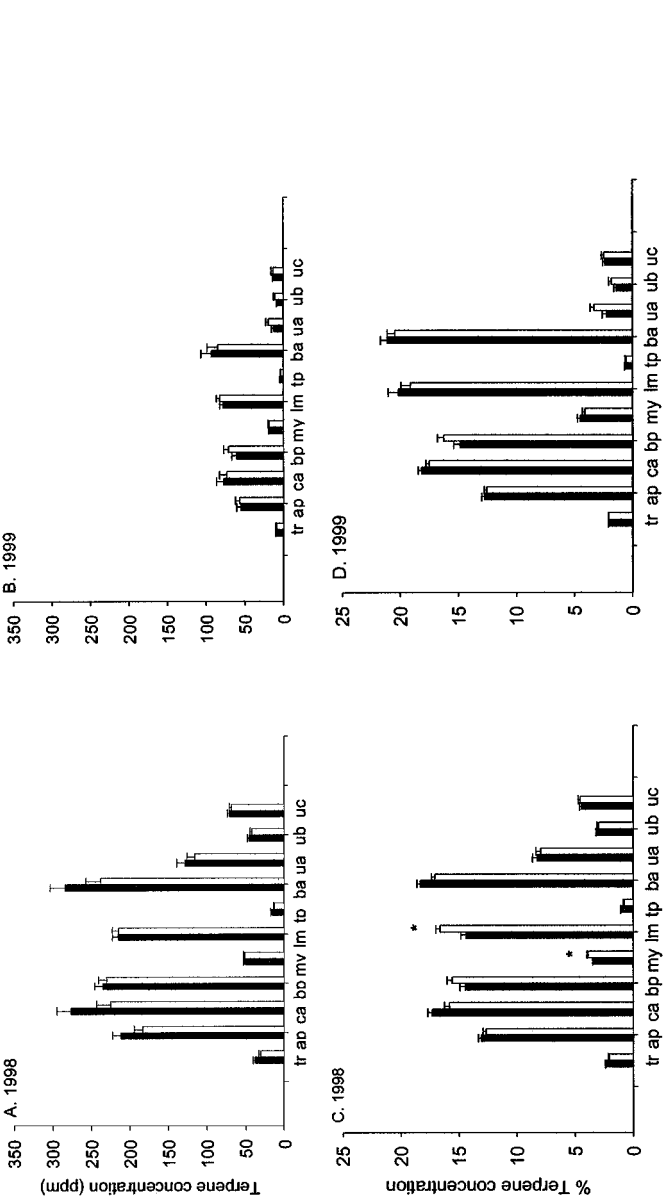


FIG. 3. Mean (+1 SE) concentration (A, B) and fractional composition (C, D) of 11 monoterpene components in Douglas fir needles sampled in the 1998 (A, C) and 1999 (B, D) greenhouse experiments for defoliated (■) versus nondefoliated (□) clones from mature Douglas fir trees (data pooled for resistant and susceptible clones). Abbreviations represented 11 monoterpenes defined in Table 1. Asterisks (*) indicate there was a significant difference between the defoliated and non-defoliated clones (ANOVA, Bonferroni adjusted $\alpha \leq 0.005$).

In the 1999 experiment, when total monoterpene concentration was 71% lower than in 1998, tree trait, defoliation treatment, and their interaction did not influence the concentration or composition of monoterpenes (MANOVA, $P \geq 0.219$; Table 2). Univariate ANOVAs also failed to detect significant effects on concentrations of individual or total monoterpenes ($P \geq 0.011$; Table 4; Figures 2B and 3B) or on the monoterpene/N ratio ($P > 0.509$). Likewise, the fractional composition of monoterpenes was unaffected by defoliation in 1999 ($P \geq 0.058$; Table 4; Figure 3D) and there were no detectable trait $\times$ treatment interactions ($P \geq 0.036$; Table 4). However, the univariate ANOVAs indicated there were significant differences between resistant and susceptible trees in the proportions of camphene, β-pinene, bornyl acetate, and unknown terpene C ($P \leq 0.005$; Table 4). Interestingly, the patterns were identical to those observed in the 1998 experiment (Figure 2C); resistant trees had higher percentages of camphene and bornyl acetate, whereas susceptible trees had higher fractions of β-pinene and unknown C (Figure 2D).

Effect of Monoterpenes and Terpene/N Ratio on Budworm Performance. In the 1998 experiment, when total monoterpene concentration was high and budworm feeding was matched to the budburst of each individual plant, budworm potential fitness declined as total monoterpene concentration increased ($P = 0.027$); approximately 20% of the variation in potential fitness was explained by total monoterpene concentration (Figure 4A). Individual terpenes that were negatively correlated with budworm fitness included myrcene ($P = 0.046$), limonene ($P = 0.029$), and unknown C ($P = 0.029$). Further, the monoterpene/N ratio was negatively related to budworm fitness in 1998 ($r = -0.481$, $P = 0.017$; Figure 4B).

In contrast, in the 1999 experiment, monoterpene concentrations were low and genetic differences among the clones in budburst phenology affected the quantity and quality of the buds and needles available for larval feeding. Under these conditions, budworm fitness was not related to total monoterpene concentration ($P = 0.567$), the fractional composition of monoterpene components (P values ranged from 0.124 to 0.954), or the monoterpene/N ratio ($P = 0.531$).

The monoterpene/N ratio was significantly higher in 1998 (0.069 ± 0.004) than in 1999 (0.011 ± 0.004) ($P < 0.001$). Although nitrogen concentration was not related to monoterpene concentration in each year, total monoterpene concentration declined exponentially as foliar N concentration increased for data pooled over both years (Figure 5). The higher monoterpene/N ratio in 1998 was caused by both higher total monoterpene concentration and lower nitrogen concentration compared to the data in 1999 (Figure 5).

Relationship between Monoterpene Concentration and Growth. Total monoterpene concentration was not correlated with the relative growth rates of the base diameter, crown, or height of the clones between the start of the experiments and the end of the 1998 growing season ($P \geq 0.814$; $N = 24$). Moreover, the five-year

TABLE 4. RESULTS FROM UNIVARIATE ANALYSIS OF VARIANCE TESTS (F TEST AND PROBABILITY VALUES) FOR EFFECTS OF TRAIT, TREATMENT, AND THEIR INTERACTION ON CONCENTRATION (FRESH-WEIGHT-BASED) AND FRACTIONAL COMPOSITION (% OF TOTAL MONOTERPENE CONCENTRATION) OF EACH MONOTERPENE COMPONENT OF DOUGLAS FIR CLONES IN 1999 EXPERIMENT WHEN BUDWORM FEEDING WAS MATCHED TO BUDBURST OF ENTIRE POPULATION OF PLANTS TO MIMIC CONDITIONS *similar to those* OCCURRING IN FOREST[a]

	Trait				Treatment (defoliation vs. nondefoliation)				Trait × treatment			
	Fresh wt		%		Fresh wt		%		Fresh wt		%	
1999 data	$F_{1,11}$	P	$F_{1,11}$	P	$F_{1,11}$	P	$F_{1,11}$	P	$F_{1,11}$	P	$F_{1,11}$	P
Tricyclene	0.62	0.449	5.78	0.035	0.00	1.000	0.04	0.839	0.70	0.421	0.42	0.529
α-Pinene	0.01	0.958	0.47	0.508	0.05	0.821	0.41	0.538	1.24	0.290	0.10	0.761
Camphene	0.17	0.691	13.57	0.004	0.08	0.788	2.54	0.139	0.48	0.502	2.92	0.116
β-Pinene	9.69	0.011	20.17	0.001	1.34	0.271	3.26	0.099	2.12	0.173	2.36	0.153
Myrcene	7.97	0.017	0.62	0.449	0.37	0.556	1.53	0.242	2.11	0.175	0.35	0.564
Limonene	6.00	0.032	0.01	0.957	0.41	0.533	0.81	0.388	4.35	0.061	0.01	0.920
Terpinolene	0.29	0.598	0.07	0.792	0.58	0.463	0.26	0.621	0.58	0.463	5.72	0.036
Bornyl acetate	0.19	0.674	12.07	0.005	0.18	0.681	0.40	0.539	0.31	0.588	0.81	0.388
Unknown A	0.41	0.536	0.02	0.888	2.38	0.151	4.46	0.058	4.43	0.059	1.05	0.327
Unknown B	0.01	0.965	1.60	0.232	1.31	0.279	1.78	0.210	2.19	0.167	0.82	0.385
Unknown C	2.56	0.138	243.32	0.001	0.24	0.631	0.17	0.691	3.57	0.086	0.10	0.756
Total	0.26	0.623			0.07	0.798			1.51	0.247		

[a] The Bonferroni adjusted P-value of $\alpha = 0.005$ (i.e., 0.05 divided by 11) was used to determine significance, in order to maintain the correct P-value of 0.05 for the type I error rate over the whole set of univariate ANOVAs for the 11 individual terpene compounds.

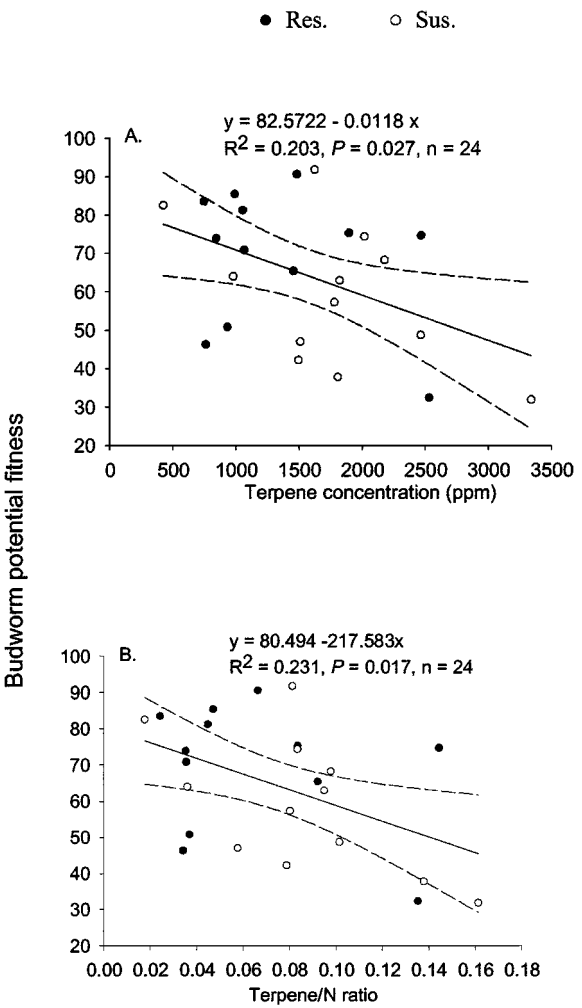


FIG. 4. Regression of western spruce budworm potential fitness against foliar concentration (ppm fresh weight) of total monoterpenes (A) and the ratio of total monoterpene concentration to foliar nitrogen (B) in the current-year foliage of clones derived from trees that appeared to be resistant (●) versus susceptible (○) to western spruce budworm defoliation. Data are from the 1998 experiment; needles were at the feather duster (seventh) developmental stage when they were sampled. Dashed lines around the regression line indicate the 95% confidence limits for the mean response, and the equations for the regression lines and their associated statistics are shown on the graphs.

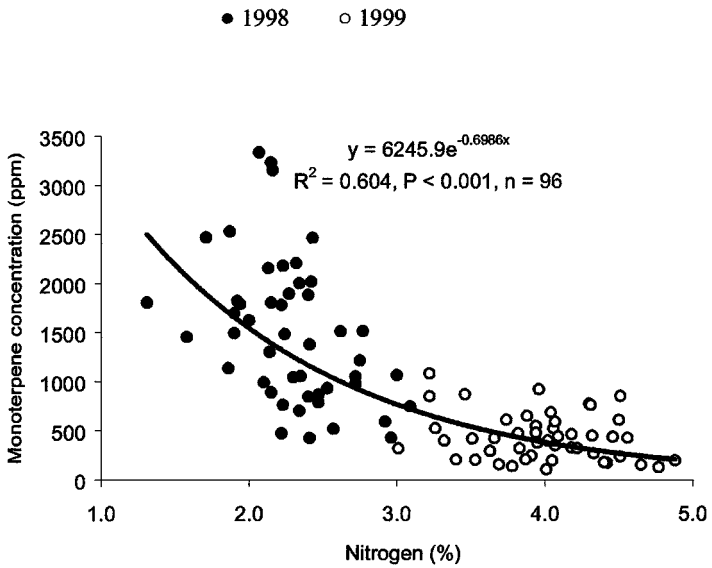


FIG. 5. Regression of foliar concentrations (ppm fresh weight) of total monoterpenes against nitrogen (% dry weight) in current-year foliage of Douglas fir clones. All needles were at the feather duster (seventh) developmental stage when they were sampled. Data are shown for the 1998 and 1999 experiments, pooled for the resistant and susceptible clones. The equation for the regression line and the associated statistics are shown on the graph.

radial growth rate of mature trees between 1986 and 1990 also was not related to the total monoterpene concentration of the mature trees in 1989 ($P = 0.902$) or 1990 ($P = 0.498$; $N = 24$).

DISCUSSION

Eleven monoterpenes were detected in current-year needles of Douglas fir clones sampled at Shepherd's (1983) feather duster (or seventh) developmental stage. They included the most common monoterpenes reported in other studies of Douglas fir, but did not include several monoterpenes, such as α-phellandrene, *cis*-β-ocimene, and 3-carene, that occurred at low concentrations in other studies (von Rudloff and Rehfeldt, 1980; Wagner et al., 1989; Zou and Cates, 1995). Compared with the relative percentage of major leaf monoterpenes of Douglas fir trees in the northern Rocky Mountains (von Rudloff and Rehfeldt, 1980), trees from southern Colorado and northern Arizona in our study had a much higher relative percentage of limonene, β-pinene, and myrcene, but a lower percentage of

tricyclene, camphene, bornyl acetate, and α-pinene. The rank order of concentration for different monoterpenes was highly correlated between the sampling years and between mature trees and their clones (Table 1), despite the variations in total monoterpene concentration over years (Figures 2A,B and 3A,B). These findings indicated remarkable consistency in fractional composition of monoterpenes.

We hypothesized that clones from resistant trees would have a higher monoterpene concentration than clones from susceptible trees based on the assumption that high monoterpene concentration would limit budworm defoliation. Testing this hypothesis with clones grown in a uniform greenhouse environment allowed us to eliminate environmental differences as a causal factor. Contrary to our hypothesis, clones from resistant trees had a lower total monoterpene concentration than clones from susceptible trees in the 1998 experiment (Figure 2A), and budworm potential fitness was greater after feeding on clones from resistant trees (Figure 4A). Thus, results from the 1998 experiment were not consistent with our field observations of resistance. This suggests that the ability of resistant mature trees to escape heavy budworm defoliation cannot be attributed to a greater genetic capacity for monoterpene biosynthesis in foliage. A difference in budburst phenology is a more likely explanation for the difference in budworm defoliation between the resistant and susceptible trees. For example, clones from resistant trees required about 90–110 more degree-days to reach the same budburst developmental stage than clones from susceptible trees (Chen et al., 2001a). This difference in budburst phenology among clones is consistent with differences in budburst phenology for mature trees in the forest (Clancy et al., 1993). Resistant trees are genetically predisposed towards later budburst that could cause poor food quality for the budworm if late budburst forces them to feed on old foliage (Clancy et al., 1993; Dodds et al., 1996).

The different results from the 1998 and 1999 experiments on the clones suggest a role of budburst phenology in regulating the influence of foliar monoterpenes on budworm performance. In 1998, we allowed budworms to feed on each clone when buds offered the best suitable food source. In this situation, genetic differences in total monoterpene concentration among clones influenced budworm fitness (Figure 4A). In contrast, in the 1999 experiment, we introduced larvae to all clones on one date when approximately 50% of the buds in the whole population were in Shepherd's (1983) second budburst stage that was just accessible for larval mining. With this approach, suitable buds available for larval feeding varied widely among clones because of genetic differences in budburst phenology (Chen et al., 2001a); the potential influence of genetic differences in monoterpenes among clones on budworm performance may have been overshadowed by differences in foliage quality associated with budburst and shoot development (Dodds et al., 1996).

Of course, an alternative explanation for the different results between the 1998 and 1999 experiments is that monoterpene concentration in 1999 was too

low to influence budworm performance (Figures 2B and 3B). This explanation implies that budworm larval performance may not be affected by total monoterpene concentration less than 1000 ppm. Nonetheless, our results raise an interesting possibility that mature trees classified as "resistant" to budworm defoliation in the forest could actually be "susceptible" in situations where the onset of budworm larval feeding coincides with their budburst. Thus, the mixed conclusions about the role of monoterpenes as a resistance mechanism against budworm defoliation in Douglas fir (Cates et al., 1983; Redak and Cates, 1984; Cates and Zou, 1990; Clancy, 1991a, 1993, 2001; Clancy et al., 1993) are perhaps not surprising, as factors such as budburst phenology may mediate the expression of this mechanism.

It is also noteworthy that there were consistent differences in 1998 and 1999 between the resistant and susceptible clones in the proportions of camphene, β-pinene, bornyl acetate, and unknown terpene C ($P \leq 0.005$; Tables 3 and 4). The resistant trees had higher percentage of camphene and bornyl acetate, whereas the susceptible trees had higher fractions of β-pinene and unknown terpene C (Figures 2C and D). This finding, to some extent, also agrees with the results of previous studies that camphene and bornyl acetate were highly toxic to several populations of budworm in Idaho, Montana, and New Mexico (Cates et al., 1983; Cates and Redak, 1988; Cates and Zou, 1990).

One or two years of budworm defoliation under our study conditions did not influence the total concentration or fractional composition of monoterpenes in either the 1998 or 1999 experiments with the clones (Figure 3). Furthermore, the large differences in total monoterpene concentration of clones (Figures 2A,B and 3A,B) and mature trees (Clancy et al., 1993) between two consecutive sample years could not be clearly explained by effects of defoliation. Moreover, defoliation had a similar effect on monoterpene concentrations of clones from resistant and susceptible trees. The lower monoterpene concentration of clones in 1999 compared to 1998 was probably caused by a decrease in tree carbon/nitrogen balance in 1999 (Figure 5) that reduced synthesis of monoterpenes and other carbon-based secondary metabolites (Bryant et al., 1983; Larsson et al., 1983; Mattson and Haack, 1987). The strong negative relationship between total foliar monoterpene concentration and nitrogen concentration for data pooled over two years of experiments support this view (Figure 5) and suggest that the carbon/nitrogen balance is important in regulating carbon allocation to monoterpenes in Douglas fir trees.

Many other traits of tree tissues can vary with budburst phenology and seasonal cycles of growth and dormancy (Mattson and Scriber, 1987; Clancy et al., 1988a,b, 1995). For example, positive correlations of growth and reproduction of insect herbivores with variation in one chemical constituent of their food do not prove cause and effect, because changes in the level of the chemical constituent in a plant may be accompanied by changes in the levels of nutrients, water, fiber, and numerous allelochemicals (Clancy, 1992). We recognize that the strong intercorrelations among many different characteristics in host plants make

it difficult to determine an herbivore's response to one specific trait, such as foliar monoterpenes. Moreover, it is impossible to maintain a constant level of monoterpenes (or other potentially confounding nutrients, etc.) throughout the duration of a greenhouse experiment because a plant's biochemical composition is continuously changing (Clancy, 1992). Thus, differences in resistance to budworm defoliation among Douglas fir genotypes are most likely caused by interactions or linkages among multiple resistance mechanisms such as monoterpenes, nutrients, and tree budburst phenology.

Finally, despite the high cost of monoterpene synthesis (Gershenzon, 1994), we found no evidence for a tradeoff between growth and monoterpene concentration in the clones and mature trees. The proportion of energy allocated to monoterpene synthesis was perhaps too small to compromise tree growth. On the other hand, perhaps high carbon allocation to monoterpenes reduced allocation to other tree uses of carbon (e.g., roots, leaves, mycorrhizae) that could not be detected by measuring stem radial growth at breast height (mature trees) or canopy size (clones).

In summary, our results suggest remarkable consistency in the percent composition of different monoterpenes in Douglas fir trees, in spite of much year-to-year variation in concentrations of these compounds. Moreover, genetic variation in budburst phenology among trees may mediate the influence of inherent differences in total monoterpene concentration on budworm fitness.

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EFFECTS OF LARGE MAMMALIAN HERBIVORES AND ANT SYMBIONTS ON CONDENSED TANNINS OF *Acacia drepanolobium* IN KENYA

DAVID WARD[1,2,4,*] and TRUMAN P. YOUNG[3]

[1]*Mitrani Department for Desert Ecology and Ramon Science Centre*
Blaustein Institute for Desert Research
Ben Gurion University of the Negev
Sede Boqer 84990, Israel

[2]*Mpala Research Center*
P.O. Box 555, Nanyuki, Kenya

[3]*Department of Environmental Horticulture*
University of California
Davis, California 95616

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Abstract—Condensed tannins have been considered to be important inducible defenses against mammalian herbivory. We tested for differences in condensed tannin defenses in *Acacia drepanolobium* in Kenya over two years among different large mammalian herbivore treatments [total exclusion, antelope only, and megaherbivore (elephants and giraffes) + antelope] and with four different ant symbiont species on the trees. We predicted that (1) condensed tannin concentrations would be lowest in the mammal treatment with the lowest level of herbivory (total exclusion), (2) trees occupied by mutualist ants that protect the trees most aggressively would have lower levels of tannins, and (3) if chemical defense production is costly, there would be a trade-off between tannin concentrations, growth, and mechanical defenses. Mean tannin concentrations increased from total exclusion treatments to wildlife-only treatments to megaherbivore + antelope treatments. In 1997, condensed tannin concentrations were significantly lower in trees occupied by the ant *Crematogaster nigriceps*, the only ant species that actively removed axillary buds. Contrary to our prediction, trees occupied by ant species that protect the trees more aggressively against mammalian herbivores did not have lower overall levels of condensed tannins. There was no consistent evidence of a trade-off between tannin concentrations and growth rate, but there was a positive correlation between mean thorn length and mean tannin

* To whom correspondence should be addressed. E-mail: dward@sun.ac.za
[4]Current address: Department of Conservation Ecology, Stellenbosch University, P. Bag X01, Matieland 7602, South Africa.

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concentrations across species of ant inhabitants and across herbivore treatments in 1997. Contrary to our expectation, trees had higher tannin concentrations in the upper parts of the canopy where there is little herbivory by mammals.

Key Words—Induced defenses, condensed tannins, *Acacia*, mammalian herbivory, ants, mutualism.

INTRODUCTION

Plants show a wide variety of defenses against herbivory, including physical responses (such as thorn production; reviewed by Myers and Bazeley, 1991), chemical defense (e.g., McNaughton and Tarrants, 1983; Du Toit et al., 1990; Bryant et al., 1992; Ward et al., 1997), growth strategies (e.g., growing too tall for the leaves to be eaten, e.g., Milewski et al., 1991; Palo et al., 1993), and low nutrient content (Lundberg and Astrom, 1990). Of increasing interest to the ecologist are inducible defenses against herbivory. When the cost of defense is high and levels of herbivory are variable, plants are predicted to have inducible defenses (Rhoades, 1979; Young, 1987; Herms and Mattson, 1992; Karban et al., 1997), i.e., they produce the defense only when herbivory is initiated so as not to commit resources prior to the need for defense. Chemical defenses are important inducible defenses because they can be produced quickly or at least transported quickly from one part of the plant to another (Bryant et al., 1983; Karban and Baldwin, 1997). There is little consensus as to the general importance of plant secondary metabolites as inducible defenses (Herms and Mattson, 1992; Karban and Baldwin, 1997) because, by definition, inducible defenses are costly to produce, yet costs of production of these chemicals have not always been detected (Bryant et al., 1983; Herms and Mattson, 1992; Simms, 1992).

Most current theories of inducible plant defenses against herbivory are based on the notion that plants will produce defenses when the benefit to be derived from them outweighs the cost of their production (e.g., Rhoades, 1979; Van der Meijden et al., 1988; Herms and Mattson, 1992; Rosenthal and Kotanen, 1994). It is generally assumed that defense is costly because investments in defense come at the expense of investments in growth and reproduction (Briggs and Schultz, 1990; Palo et al., 1993; reviewed in Herms and Mattson, 1992). Due to the costs of defense, it is also frequently assumed that few plants invest simultaneously in more than one mechanism of herbivore deterrence and that defense traits are negatively correlated (reviewed by Zangerl and Bazzaz, 1992; Silvertown and Dodd, 1996).

African Acacias as Model Organisms for Tests of Inducible Defenses. African acacias evolved as savanna trees under intense pressure by mammalian herbivores (Ross, 1979; Scholes and Walker, 1993). *Acacia* trees have long thorns and high polyphenol (especially condensed tannin) contents, both of which are effective in deterring mammalian herbivores (Wrangham and Waterman, 1981; Cooper and

Owen-Smith, 1985, 1986; Furstenburg and van Hoven, 1994). Condensed tannins are generalized digestion inhibitors in mammals (Robbins et al., 1987; Waterman and Mole, 1994). Although condensed tannins may also be effective against insect attack, the importance of these organisms as agents of selection on defenses of African *Acacia* species is generally considered to be low (Cooper and Owen-Smith, 1985; Du Toit et al., 1990; Owen-Smith, 1993; Gowda, 1997; Rohner and Ward, 1997). Inducible thorn defenses against herbivory in *A. drepanolobium*, *A. seyal* (Young, 1987; Milewski et al., 1991), *A. tortilis* (Gowda, 1996a; Rohner and Ward, 1997), and *A. raddiana* (Rohner and Ward, 1997), as well as inducible polyphenol defenses in *A. raddiana* (Rohner and Ward, 1997), suggest that defenses are costly in *Acacia* species. This conclusion is reinforced by the experimental evidence that investment in thorn defenses in *A. drepanolobium* declined following removal of mammalian herbivores (Young and Okello, 1998). Thus, *Acacia* species may be model organisms for investigating the inducibility of defenses against mammalian herbivory. Furthermore, because they frequently have long thorns and high tannin concentrations, they may be suitable species for testing for the presence of possible trade-offs between investments in different types of defense.

Plants may concentrate their defenses in those structures or life-history stages that are most likely to be affected by herbivory (e.g., Palo et al., 1993). The most clear-cut division between accessible and nonaccessible parts of trees depends on the height of the tree relative to the maximum browsing height of nonarboreal herbivores (Palo et al., 1993; Gowda, 1996a). Younger *Acacia* trees have higher levels of mechanical defense and chemical defenses than older trees (Palo et al., 1993; Brooks and Owen-Smith, 1994; Gowda, 1996b; Rohner and Ward, 1997), and within trees, lower branches have longer thorns than higher branches (Young, 1987; Milewski et al., 1991; Young and Okello, 1998). This suggests that plants and plant parts that are more exposed to mammalian browsers invest more in defense, while larger trees normally escape mammalian herbivory and, thus, allow for more varied and flexible investments (including reproduction) and only produce stronger defenses when necessary (see also Brooks and Owen Smith, 1994; Rohner and Ward, 1997). Here, we investigate the effects of mammalian herbivory on condensed tannins in a common African *Acacia*, *A. drepanolobium*, and determine whether the level of defense is related to the probability of herbivory.

Ants as Acacia Defenses. Ants that live in the swollen thorns of *Acacia* species are frequently assumed to provide a form of defense against large mammalian herbivores because they swarm over the herbivores and bite them when they come into contact with the trees (e.g., Hocking, 1970; Coe and Beentje, 1991). Madden and Young (1992) showed that *A. drepanolobium* with more active ants had more foliage than did trees with less active ants and were less heavily browsed by immature (but not mature) giraffes.

Although several African *Acacia* species produce swollen thorns and are occupied by ants, *A. drepanolobium* is the only species known to be an obligate ant

TABLE 1. ANT SPECIES COMMONLY RESIDENT ON *Acacia drepanolobium* AND THEIR ROLES,[a] SUCCESSIONAL STAGE, AND EFFECT ON THE PLANT

Species	Interaction with tree	Successional stage	Ant behavior	Feeds on
Crematogaster mimosae	Mutualist	Late	Aggressive	
C. sjostedti	Non-Mutualist	Late	Timid	
C. nigriceps	Non-Mutualist	Early	Aggressive	Axillary shoots
Tetraponera penzigi	Non-Mutualist	Early	Timid	Extrafloral nectaries

[a] Data from Young et al. (1997).

host (Ross, 1979). Several ant species on *A. drepanolobium* are mutually exclusive (Hocking, 1970), and certain ant species appear to replace others in a successional process (Young et al., 1997) (Table 1). Two of the ant species found on *A. drepanolobium* respond aggressively when their branches are disturbed (*Crematogaster mimosae* and *C. nigriceps*). Because aggressive ants may reduce the threat of herbivory to the trees (Madden and Young, 1992), we predicted that there would be a reduction in tannin concentration in trees occupied by more aggressive ants. Two less aggressive ant species on *A. drepanolobium*, *C. sjostedti* and *Tetraponera penzigi*, may be less effective at protecting their trees from herbivory (Table 1) (Young et al., 1997). In addition, two species of ants have unusual behaviors. *C. nigriceps* ate virtually all of the axillary shoots at nodes with unswollen thorns on trees they occupied (Young et al., 1997). Unlike *A. drepanolobium* inhabited by other ant species, few trees occupied by *C. nigriceps* flower or set fruit. Trees occupied by *T. penzigi* were 2–9 times more likely to have at least some leaves with their nectaries eaten compared to trees without *T. penzigi*. Most of the nectaries were eaten on these trees and more than 90% of them lacked any active nectaries, whereas only 26–38% of other trees lacked active nectaries (Young et al., 1997).

The potential interactions between the effects of mammalian herbivory and ant occupancy on *A. drepanolobium* provide a unique opportunity to examine the relative effects of mammalian herbivores and ant mutualists on plant chemical defenses. We simultaneously examined the relative effects of mammalian herbivory and ant occupancy on tannin production in an experimental design with replicated 4-ha plots that either allowed (1) the entry of all large mammalian herbivores, (2) all large mammalian herbivores except megaherbivores (elephants *Loxodonta africana* and giraffe *Giraffa camelopardis*) to enter, or (3) excluded all large herbivores (Young et al., 1998). Within each of these plots, we sampled trees with each of the different ant occupants listed in Table 1.

We made the following predictions about condensed tannin concentrations in *A. drepanolobium*:

1. Trees from plots with higher levels of mammalian herbivory should have higher concentrations of condensed tannins in their leaves to defend themselves

against herbivory. Thus, the highest tannin levels should be found in the megaherbivores + antelope plots, followed by the antelope plots, and then the total exclusion plots.

2. More aggressive ant species (*C. nigriceps* and *C. mimosae*) may protect their trees from herbivory more than less aggressive species (*C. sjostedti* and *T. penzigi*). This should result in reduced tannin concentrations in the leaves of trees occupied by mutualist ant species.

3. Because inducible defenses are hypothesized to be costly to produce (Herms and Mattson, 1992; Zangerl and Bazzaz, 1992), there should be a trade-off between investments in chemical (tannins) and physical (thorn) defenses. Similarly, there may be a trade-off between growth rate and chemical defenses.

4. If *A. drepanolobium* is capable of investing differentially in different parts of the canopy according the probability of being eaten, we should find lower tannin levels high in the upper canopy where mammalian herbivory is low, with this effect being reduced in the megaherbivore plots.

METHODS AND MATERIALS

Research was conducted at the Mpala Research Centre in the Laikipia ecosystem (36°50′E, 0°15′N) in north-central Kenya. *Acacia drepanolobium* is the dominant tree species in the wooded grasslands on "black cotton" soils in this ecosystem, comprising approximately 95% of woody individuals (Young et al., 1997, 1998). There are 18 experimental plots (fenced in September 1995), each 4 ha in size and located in three blocks. Within each block, different forms of barriers and control of individual cattle herds create six mammalian herbivore treatment levels. The treatment levels are: (1) total exclusion, (2) cattle only, (3) nonmegaherbivore wildlife (antelope) only, (4) nonmegaherbivore wildlife (antelope) + cattle, (5) all wildlife, including megaherbivores (= elephants + giraffe) and antelope, and (6) all wildlife (megaherbivores and antelope) + cattle. This design has one replicate per block. Because cattle do not eat *Acacia* leaves (Young and Okello, 1998), we collapsed the treatment levels into three wild mammalian herbivore levels, allowing two replicates per block. The treatment levels we considered here were total exclusion, antelope, and antelope + megaherbivores (elephants and giraffes). Browsing species that occurred in the antelope plots were: Grant's gazelle (*Gazella granti*), eland (*Taurotragus oryx*), steinbuck (*Ramphicerus campestris*), and oryx (*Oryx beisa*). Antelope plots also included zebras, buffaloes, and hartebeests, but these grazers do not eat *Acacia* leaves and will not concern us here. Densities of large mammals were not controlled within the plots other than by restricting access (Young et al., 1998). Dung counts suggest that the antelope barriers reduce the presence of antelopes by 90% and that the megaherbivore barriers reduce the presence of elephants and giraffes by >80% (Young et al., 1998).

In July 1997, within each of the 18 plots, we collected undamaged leaves over a short time period approximately 1 m above the ground of one tree for each of the four ant species mentioned above. We sampled leaves at two heights (1 and 2 m) on trees occupied by the mutualist ant species, *C. mimosae*. In all but two cases, these were different trees. This procedure was repeated in 1998 on 90 new trees, each paired with a nearby tree from 1997 with respect to ant occupant and branch height. These 1998 trees also served as control trees for a leaf removal experiment.

The branches from which we sampled leaves in 1997 were also the basis of a leaf removal experiment. Each branch selected was randomly chosen from a simple forked pair of branches of similar length. After collecting leaves for our tannin analysis, we removed all remaining leaves from the leaf removal branch, while keeping its paired branch as a control.

In combination with the new trees sampled in 1998, this experiment had three levels: leaf removal, leaf control on the same tree, and (a separate) control tree. If trees invest preferentially in sections that have had leaves removed, then tannin concentrations should be highest in the leaf removal treatments in the following year. If tannins are invested at the level of the tree as a whole, leaf removal and leaf controls should have similar tannin concentrations that will be higher than in the control trees.

We also attempted to ascertain whether there were trade-offs between tannin production and investments in thorns and in growth. Mean thorn (=spine) lengths across multiple trees occupied by different ants species in these plots were taken from Young and Okello (1998). We also had mean thorn length measurements from a small number of thorns on each sampled tree. Growth rates of individual branches were measured as the difference between branch lengths in surveys in 1997 and 1998.

Leaves were air-dried prior to measurement of condensed tannins. All analyses were done simultaneously. We measured condensed tannins in the leaves using a standard acid–butanol protocol (Waterman and Mole, 1994). Following the recommendation of Hagerman and Butler (1989), quebracho was used as a standard (quebracho supplied by A. E. Hagerman, Miami University, Ohio, USA). All results are expressed as percent dry weight in quebracho equivalents (QE).

The data were analyzed in a randomized block ANCOVA with mammal treatment as a fixed factor and ant species and branch height class as random factors (error terms as for model III ANOVA). Height of tree and length of new growth on the sampled branch were used as covariates.

RESULTS

There was no correlation in condensed tannin concentration within trees between years ($r = 0.15$, $F = 2.14$, $P = 0.15$). For 89 unmanipulated trees for which we had data in both 1997 and 1998, we found no difference in tannin

TABLE 2. TANNIN CONCENTRATIONS IN 1997 AND 1998 ON TREES INHABITED BY *C. mimosae*[a]

Branch height	Mammalian treatment	Herbivory	Tannin concentration (% QE)
Low (1m)	Control	None	16.27 ± 1.63
	Antelope only	High	10.96 ± 1.83
	Megaherbivores + antelope	High	13.44 ± 2.43
High (2m)	Control	None	19.29 ± 1.59
	Antelope only	None	17.56 ± 2.12
	Megaherbivores + antelope	Low	17.12 ± 1.07

[a] Estimates of herbivore presence are based on dung counts of herbivores that can reach reference branches.

concentration between years (paired t test, $t = 0.76$, $P = 0.45$). In no analysis were there significant year effects. There were also no block effects. Overall, there was tremendous variation in tannin concentrations among samples (range 2–30% QE, coefficient of variation = 37%).

We only sampled higher branches on trees occupied by *Crematogaster mimosae* ($N = 47$ trees). Tannin levels from upper branches were 33% higher than on lower branches, even after controlling for the identity of ant occupant by using only trees occupied by *C. mimosae* (Table 2). This was true for both years, for all herbivore treatments, and in both experimental and control trees and branches. For all of the analyses that follow, upper and lower branches were analyzed separately, or only lower branches were analyzed.

Among lower branches, there were no differences in condensed tannin concentration among trees occupied by different ant species. However, there was a difference in condensed tannins among 1997 trees with different occupant ant species ($P = 0.03$, Table 3). Contrary to prediction 2, the lowest tannin levels were found in trees with a nonmutualist species, *C. nigriceps*, while trees with *C. mimosae*, a mutualist species, had the highest tannin levels (Figure 1). Scheffé *post hoc* tests revealed that there was a difference in tannin concentrations between

TABLE 3. PARTIAL TABLE FROM RANDOMIZED BLOCKS ANCOVA FOR SUMMER 1997 DATA[a]

Source	*d.f.*	*F*	*P*
Block	2	1.20	0.31
Herbivores	2	0.36	0.87
Ant species	3	3.08	0.03
Herbivores × ant species	6	1.03	0.44
Tree height	1	3.87	0.05
Branch height	1	15.24	<0.001

[a] Dependent variable is condensed tannin concentration.

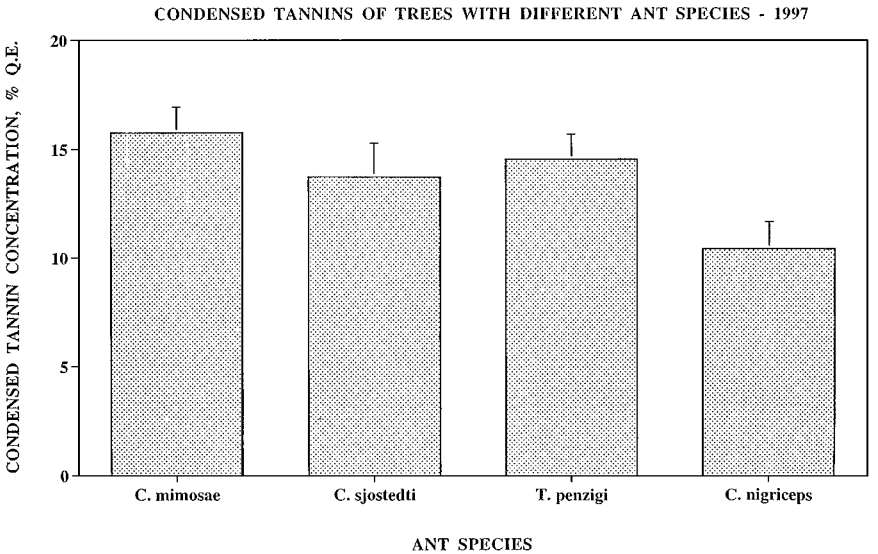


FIG. 1. Differences in mean condensed tannin concentrations (+SE) in *A. drepanolobium* among ant occupants for unmanipulated trees in 1997.

trees occupied by *C. nigriceps* and *C. mimosae* ($P = 0.03$) only. These two species swarmed more aggressively than *Tetraponera penzigi* or *C. sjostedti*. In 1998, there was no difference in condensed tannins among trees with different occupant ant species ($F = 0.67$, $P = 0.58$).

We found no effect of our leaf removal treatments ($F = 1.71$, $P = 0.18$). Paired t test comparisons that either controlled for within-tree variation (leaf removal vs. leaf control) or paired leaf removal vs. control tree or leaf control vs. control tree were not significant (range of $t = 0.22$–1.20, $P = 0.83$–0.23). Condensed tannin concentration in control tree leaves was 14.80 ± 0.64 (S.E.) % QE ($N = 89$), in leaf controls was 13.65 ± 0.74% QE ($N = 74$), and in the leaf removal treatment was 12.87 ± 0.83% QE ($N = 75$). There were no interactions between leaf removal treatments and either herbivore treatments ($F = 0.41$, $P = 0.80$) or ant treatments ($F = 0.67$, $P = 0.67$).

When both years were combined (180 different trees, no tree measured twice), there were no year or ant species effects, but there was a significant mammalian herbivore effect (Table 4). There appears to be a difference in these data between the confirmed mutualist (*C. mimosae*) and the other ants in terms of how tannin concentrations respond to mammalian herbivore treatments, resulting in a significant ant $\times$ herbivore treatment interaction ($F = 2.35$, $P = 0.006$). This interaction means that for trees without *C. mimosae*, the differences between mammalian herbivore treatments are consistent with prediction 1 ($P = 0.05$;

TABLE 4. PARTIAL TABLE FOR REDUCED ANOVA MODEL FOR ALL DATA FOR BOTH YEARS USING UNMANIPULATED TREES ONLY[a]

Source	*F*	*P*
Year	0.95	0.33
Herbivores	3.11	0.05
Ant species	2.08	0.13
Ant × year	1.89	0.16

[a] Dependent variable is condensed tannin concentration. The interaction term was included in this analysis because in the full ANOVA model with all interaction terms, this was the only interaction with $P < 0.25$. There were also no significant block effects ($P = 0.91$) in the full model.

Control $= 12.03 \pm 0.89\%$ QE, antelope only $= 12.98 \pm 1.07\%$ QE, megaherbivores + antelope $= 15.24 \pm 0.88\%$ QE). Data for each of the three ant species separately show this trend.

Within *C. mimosae* trees, the results are essentially reversed. Tannin levels are high on branches exposed to little or no herbivory (i.e., contra prediction 4), and low on branches subject to high herbivory (Table 2). We identified dung piles according to mammalian herbivore species and grouped these species according to whether they were tall enough to reach either the 1-m or 2-m branch height classes. Among individual plots, tannin levels were negatively correlated with the number of dung piles of species capable of reaching either the 1-m or 2-m branch height classes ($F = 5.82$, $P < 0.02$).

There was no correlation between tree height and tannin concentrations ($r = 0.11$, $F = 1.08$, $P = 0.30$) in 1997, although there was a positive correlation between branch height and tannin concentrations ($r = 0.37$, $F = 14.22$, $P < 0.001$).

Trade-Offs. Tannin concentration and thorn length were not correlated in 1997 ($r = 0.20$, $F = 2.61$, $P = 0.11$) or 1998 ($r = 0.074$, $F = 0.78$, $P = 0.38$). There was also no interaction effect between tannin concentration, thorn length, and either ant species (1997: $F = 0.29$, $P = 0.83$; 1998: $F = 0.39$, $P = 0.76$) or herbivore treatment (1997: $F = 0.51$, $P = 0.60$; 1998: $F = 0.15$, $P = 0.86$). When data for each ant species were analyzed separately, there was no correlation between tannin concentration and thorn length in 1997 and 1998 (range of $F = 0.06$–0.85, range of $P = 0.81$–0.25). However, contrary to prediction 3, greater mean tannin concentration was associated with greater mean thorn length across ant species in 1997 ($r = 0.99$, $F = 202.6$, $P = 0.005$) (Figure 2), but not in 1998 ($r = 0.15$, $F = 0.04$, $P = 0.85$).

For low branches (height = 1 m), and with data for each ant occupant species treated separately, we found a significant correlation between tannin concentration

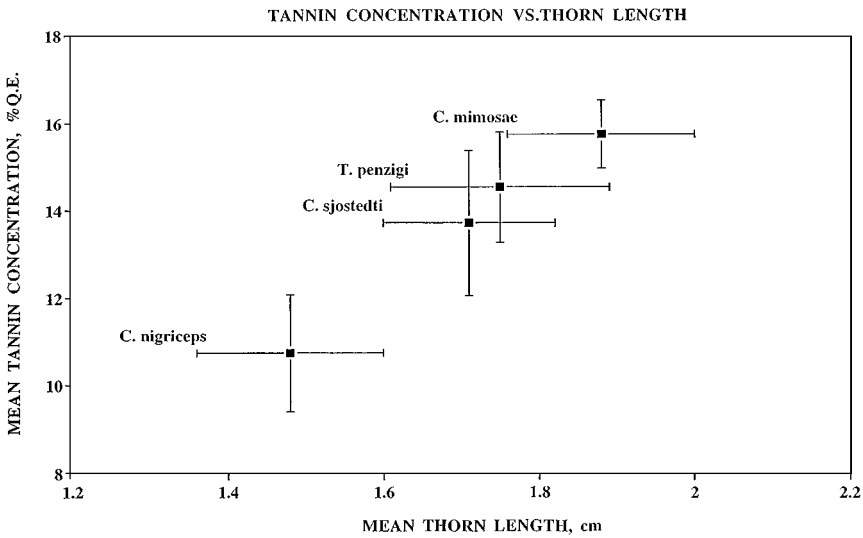


FIG. 2. Relationship between mean condensed tannin concentration and mean thorn length per tree averaged across ant treatments. Error bars represent ±1 SE for both tannin concentration (ordinate) and thorn length (abscissa).

and new branch growth for trees occupied by *T. penzigi* only. However, this correlation was positive ($r = 0.55$, $F = 11.34$, $P = 0.002$, $N = 28$). For high branches (height = 2 m), we had data for trees occupied by *C. mimosae* only. There was a nonsignificant negative trend between tannin concentration and branch growth, but this was not significant ($r = -0.33$, $F = 3.37$, $P = 0.08$, $N = 29$). Thus, there is no support for prediction 3, viz. that there is a trade-off between tannin production and growth.

DISCUSSION

Inducible Defenses. There was considerable variation in condensed tannin concentrations among *A. drepanolobium* individuals and in different parts of the canopies of individual trees. Similarly, there was high variation in thorn lengths within and among individuals (Young and Okello, 1998), which means that substantial sample sizes are necessary to reveal even moderate effect sizes. Some of the variation in tannin concentrations includes interyear differences within trees and differences among mammalian and ant treatments.

Our pooled results for 1997 and 1998 provide evidence for inducible tannin defenses across mammalian herbivore treatments in a manner that is consistent with prediction 1. However, this effect was small and was revealed only after

excluding trees occupied by one of the ant species. The absence of a strong effect of herbivore treatments may be due to the short time available for the production of inducible defenses (see also Young and Okello, 1998) or because these trees do not strongly respond to mammalian herbivory. In addition, tannin response to herbivory may be ephemeral (Furstenburg and van Hoven, 1994).

An additional consideration is that secondary compounds may not always be effective against mammalian herbivores. The condensed tannin concentrations found in *A. drepanolobium* in this study were relatively low, and it is uncertain whether they reached a threshold level necessary to deter ruminants as suggested by Cooper and Owen-Smith (1985). Further examination of the behavioral and physiological responses of local mammalian herbivores to different levels of tannin production is required to answer this question. We believe that the search for generalities regarding the inductive responses of trees to mammalian herbivory must necessarily focus on the abilities of the trees to respond in a timely manner and with tannin levels of sufficient concentration to act as a deterrent to further herbivory. Futhermore, optimal tree response strategies with regard to growth–defense relationships, as well as possible trade-offs between multiple defensive strategies (e.g., tannins and thorns), must be reconsidered (see below).

Ant occupants appeared to exert a greater effect on condensed tannins than mammalian herbivore treatments. It is possible that differences among ant treatments occurred because ants choose trees according to their tannin phenotypes. We consider this to be unlikely because ant species replace one another on trees (Young et al., 1997). Trees occupied by the two more aggressive ant species did not have lower tannin concentrations than trees occupied by the less aggressive ant species. This result is inconsistent with prediction 2. Trees occupied by one of the two aggressive species, *C. nigriceps*, did exhibit lower tannin levels (at least in 1997); this is the ant species that eats the axillary shoots of its host trees, and reduces the number of leaves per branch. One possibility is that the negative impact of the ant on the leaves reduces the resources available to the tree to invest in tannin defenses. An alternative explanation is that their destruction of axillary buds results in the production of larger, more nectariferous (observed), and physiologically younger (implied) leaves. "Younger" leaves may produce less tannin (Furstenburg and van Hoven, 1994).

There was also a significant ant $\times$ herbivore treatment interaction, with trees occupied by *C. mimosae* exhibiting a pattern in which shoots subject to less herbivory contained substantially more tannin than shoots subjected to more herbivory. This contradicts both prediction 1 and prediction 4. It is also curious that the antelope-only treatment had lower tannin concentrations than the total exclusion plots in 1998. Are there lower levels of tannins where herbivory is highest under certain circumstances because browsing causes a local decline in tannins? At first sight, such results might seem counterintuitive because it is usually assumed that tannins are produced in order to reduce herbivory and might be expected to

increase in concentration under high herbivory. However, other studies of African acacias have found that tannin concentrations decreased with herbivory. Du Toit et al. (1990) found that heavily browsed specimens of *A. nigrescens* had greatly reduced condensed tannin concentrations and may serve as "grazing lawns" (sensu McNaughton, 1983) for herbivores. They proposed the following mechanism for this phenomenon: Browsing reduces intershoot competition for nutrients through a reduction in the number of shoots and the release of apical dominance. The result is increased concentration of nutrients in the remaining shoots. Increased nutrient concentrations in the remaining shoots was recorded by them for *A. nigrescens* [but not *A. tortilis*; see also Gowda (1997)] and Teague (1989) for *A. karroo.* Du Toit et al. (1990) also showed that there was a strong positive correlation in *A. nigrescens* between leaf nitrogen concentration and the percentage of browsed shoots on a tree. This increased nutrient concentration, together with a release of apical dominance, stimulates growth. We note here that Young et al. (1997) found that *A. drepanolobium* trees occupied by *C. nigriceps* showed greater shoot growth than trees occupied by the other ant species. Furthermore, carbohydrate demands incurred by rapid regrowth cause substrate limitation of carbon-based secondary metabolite synthesis (Bryant et al., 1983, 1987; Danell and Huss-Danell, 1985). Hence, leaf condensed tannin concentrations decline. Reduced chemical defense and increased shoot nutrients increase the palatability of most woody plants (Bryant et al., 1983; Cooper and Owen-Smith, 1985; Danell and Huss-Danell, 1985; Cooper et al., 1988), thereby attracting increased browsing pressure. This hypothesis remains to be tested. We note here that another study (Rohner *and* Ward, 1997) found no reduction in secondary compounds in *A. raddiana* and *A. tortilis* that suffered unnaturally high levels of herbivory in Israel.

Our data certainly suggest that tannin responses are highly contingent effects. We tentatively suggest that some forms of browsing may sufficiently increase carbohydrate demands for regrowth and cause a trade-off with secondary metabolite synthesis and, thus, result in a subsequent decline of tannin concentrations. If browsing is not severe enough, or does not result in the release of apical dominance, intershoot competition will not be reduced and the process will not occur as outlined by Du Toit et al. (1990), and induced production of tannins may then occur in trees suffering herbivory.

Trade-Offs. There was no indication of trade-offs between investments into chemical or mechanical defense or between chemical defense and growth in *Acacia drepanolobium*. Indeed, across trees occupied by different ant species, mean tannin concentrations and thorn lengths were positively correlated in 1997 (Figure 3), in contradiction with prediction 3. Several issues arise concerning underlying assumptions when attempting to demonstrate trade-offs between thorn and tannin defenses in individuals within a population (see also Rohner and Ward, 1997):

1. Chemical and mechanical defenses may not be operating at a comparable time scale. Production of thorns is irreversible and may integrate a response over

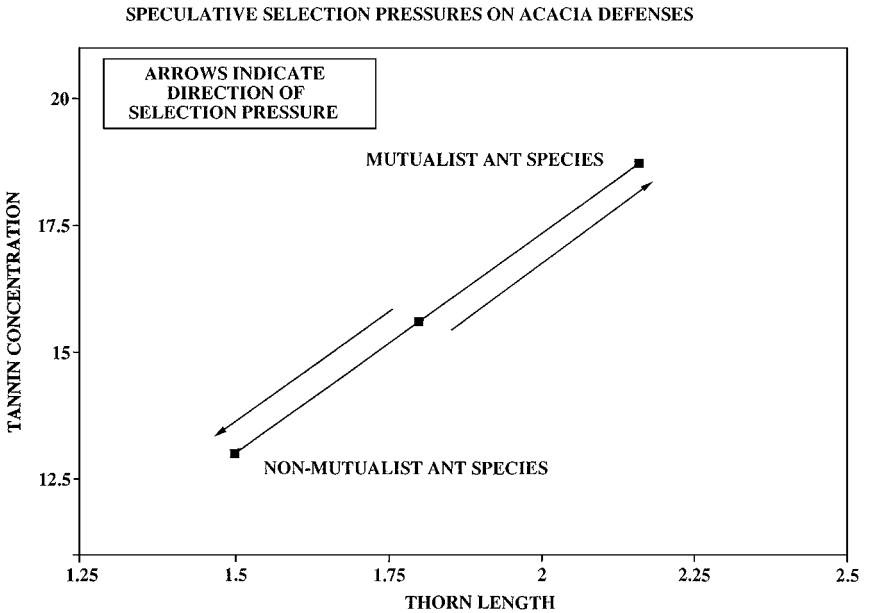


FIG. 3. Schematic diagram indicating how bidirectional selection by mutualist and non-mutualist ant species might result in a positive correlation between tannin and thorn production.

several years. Production of secondary metabolites, in contrast, is more variable on a shorter time scale (Cooper et al., 1988; Furstenburg and van Hoven, 1994). Indeed, in this study we found no significant correlations in tannin concentrations among years within trees. This difference in time scale between the production of thorn and chemical defenses may obscure correlations.

2. Phenotypic changes in response to herbivory can cause positive, rather negative, correlations. The negative impact of *C. nigriceps* on the trees (reduced leaf area and virtually eliminating reproduction) may cause the trees to be stressed, reducing their abilities to invest in both tannins and thorns. Conversely, a mutualist species such as *C. mimosae* may increase the tree's quality (e.g., by reducing herbivory), resulting in more resources being available to invest in both tannins and thorns against mammalian herbivory. These effects may result in bidirectional selection (Figure 3), causing the observed positive correlation.

3. If there is a small difference between good and poor individuals in the amount of resources acquired (allocated to total defense) and a large difference in the relative allocation of resources to tannin and thorn defenses, then a trade-off will occur (Van Noordwijk and de Jong, 1986) (Figure 4a). If there is a large difference between good and poor individuals in the amount of resources acquired (allocated

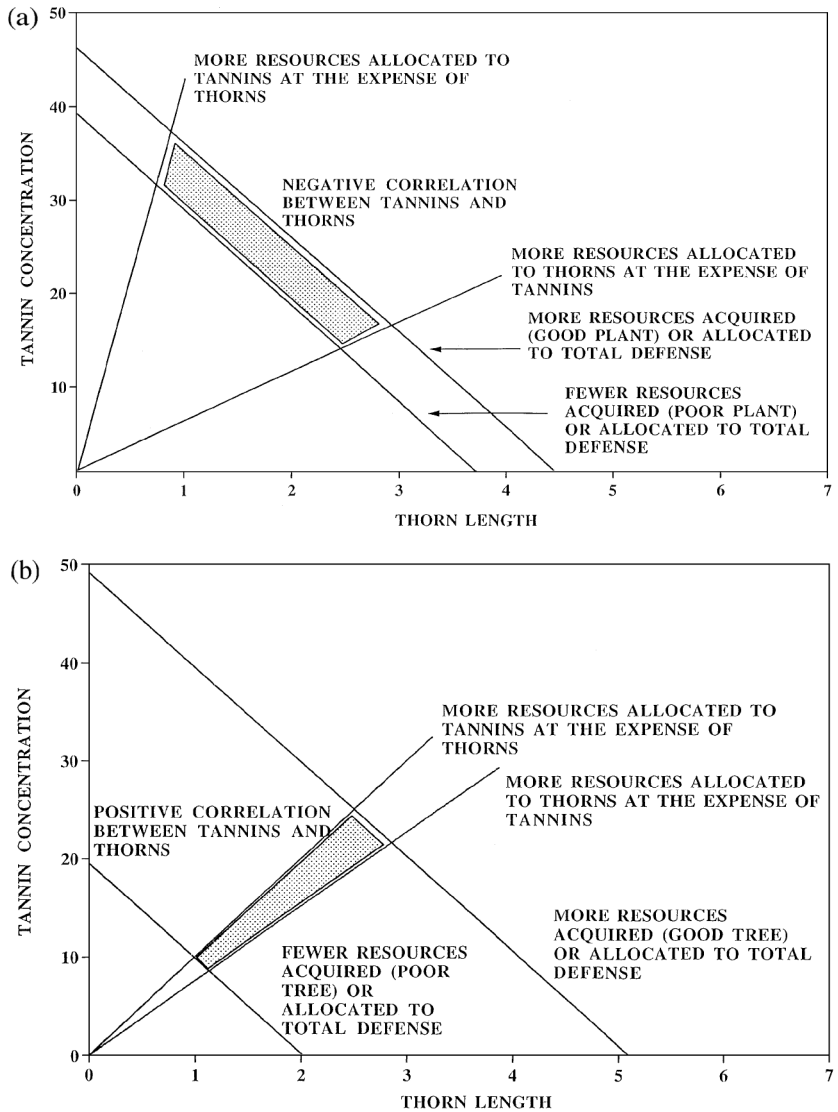


FIG. 4. (a) Schematic diagram indicating how a small difference between good and poor individuals in the amount of resources acquired (or allocated to total defense) and a large difference in the relative allocation of resources to tannin and thorn defenses will cause a trade-off (Van Noordwijk and de Jong, 1986). (b) Schematic diagram indicating how a large difference between good and poor individuals in the amount of resources acquired (or allocated to total defense) and a small difference in the relative allocation of resources to tannins and thorns will result in a positive correlation (Van Noordwijk and de Jong, 1986).

to total defense) and a small difference in the relative allocation of resources to tannins and thorns, a positive correlation will result (Van Noordwijk and de Jong, 1986) (Figure 4b).

The scenarios in Figures 3 and 4b are not mutually exclusive, i.e., large differences in resource availability may be induced by the activity of the ants which, if combined with low variability among plants in resource allocation, will result in the scenario outlined in Figure 4b. Further experimentation, such as ant removals combined with manipulation of resource availability, is needed to ascertain whether these explanations are appropriate for *Acacia drepanolobium*.

4. Defenses may not be costly to the plant. Inducible defenses were apparent for physical defenses in *A. drepanolobium*, *A. seyal* (Young, 1987), *A. tortilis* (Gowda, 1996a; Rohner and Ward, 1997), and *A. raddiana*, suggesting that thorn production is costly and resources are allocated differently when browsing levels are low. However, the results of Rohner and Ward's (1997) study on *A. tortilis* in Israel, Gowda's (1997) study on *A. tortilis* in East Africa, and the study by Du Toit et al. (1990) of *A. tortilis* in South Africa did not confirm this for secondary compounds.

Thus, it still is not clear whether condensed tannins can be considered as general defense compounds in *Acacia* species, whether they are effective inducible defenses (see review by Owen-Smith, 1993), and under which conditions such responses are likely to be expressed. Further research is needed to understand these relationships. Our original, admittedly simplistic, view of these compounds as defenses against herbivory certainly needs revision. Undoubtedly, such revision will need to incorporate more realistic models of physiological constraints to defense production and its relationship with investments in other traits such as growth.

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DOES DECREASED MOWING FREQUENCY ENHANCE ALKALOID PRODUCTION IN ENDOPHYTIC TALL FESCUE AND PERENNIAL RYEGRASS?

SEPPO O. SALMINEN* and PARWINDER S. GREWAL

Department of Entomology
OARDC/The Ohio State University
Wooster, Ohio 44691-4096

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Abstract—Tall fescue, *Festuca arundinacea*, and perennial ryegrass, *Lolium perenne*, are widely infected with fungal endophytes (*Neotyphodium* spp.). The symbiosis between plant and fungus leads to synthesis of alkaloids that have been shown to be either toxic or act as feeding deterrents against insect pests. As cultural practices have the potential to regulate production of plant secondary metabolites, we evaluated the influence of mowing frequency on the levels of major alkaloids in tall fescue and perennial ryegrass in the greenhouse. Tall fescue and perennial ryegrass maintained in 15-cm-diam. pots were cut to 5-cm height weekly or biweekly. Samples were taken monthly and the alkaloids extracted and analyzed by reverse-phase LC-MS. In tall fescue, ergovaline, ergonovine, and ergocristine were identified, whereas only ergocristine was identified in perennial ryegrass samples. In tall fescue, we observed a trend showing higher levels in samples cut biweekly than in those cut weekly. A similar pattern was seen in some putative alkaloids that were not identified. In perennial ryegrass, ergocristine and two putative alkaloids followed a pattern similar to that of alkaloids in tall fescue. A survey of a few samples of perennial ryegrass using extractions specific to peramine and lolitrem B yielded evidence suggesting their presence as well as several other identified alkaloids. These data support the hypothesis that decreased mowing frequency enhances alkaloid production/accumulation in tall fescue and perennial ryegrass.

Key Words—Fungal endophytes, *Neotyphodium coenophialum*, *Neotyphodium lolii*, alkaloids, tall fescue, *Festuca arundinacea*, perennial ryegrass, *Lolium perenne*, mowing frequency.

* To whom correspondence should be addressed. E-mail: salminen.1@osu.edu

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INTRODUCTION

Tall fescue, *Festuca arundinacea* Schreb, and perennial ryegrass, *Lolium perenne* L., are widely infected by the fungal endophytes *Neotyphodium coenophialum* Morgan-Jones & Gams and *Neotyphodium lolii* Latch, Christensen & Samuels, respectively. These mutualistic symbioses lead to production of several alkaloids that provide infected plants with chemical defenses against herbivory (Prestidge et al., 1982). Livestock feeding on the endophyte-infected grass suffer toxicoses from alkaloid ingestion (Porter et al., 1974; Hoveland, 1993). At least 15 species of insects are affected by endophyte alkaloids (Siegel et al., 1990; Rowan et al., 1990; Breen, 1993). This chemical defense also extends to other organisms including nematodes (West et al., 1988) and fungi (White and Cole, 1985).

Tall fescue and perennial ryegrass are widely used turf grasses in home lawns, golf course fairways, driving ranges, and public parks. They sometimes require application of pesticides to manage insect pests. Heavy use of chemical pesticides in urban environments leads to increased health risks to pets, children, and adults. Not surprisingly, the use of many organophosphate and carbamate pesticides has been banned by the United States Environmental Protection Agency or is under review in order to implement the Federal Food Quality Protection Act. However, this has, led to a more intensive use of other pesticides, such as neonicotinoids, that are deemed safe, relative to organophosphates.

The potential of endophyte-mediated resistance offers a promising avenue of investigation (Clay, 1989). If pesticide use in urban environments could be reduced through cultural practices (fertilization, irrigation, clipping), this could lead to health and economic benefits for homeowners and managers of golf courses and recreational parks. There are indications that the alkaloid levels in endophyte-infected turf grasses can be manipulated by management practices. For instance, clipping has been shown to reduce alkaloid content in tall fescue (Belesky and Hill, 1997). This occurred as a function of nonstructural carbohydrates that were channeled from alkaloid synthesis to production of leaf matter. This led us to hypothesize that decreased mowing frequency would result in an increase in the level of endophytic alkaloids. Here we present results of a greenhouse study on the effect of mowing frequency on levels of major alkaloids produced in tall fescue and perennial ryegrass.

METHODS AND MATERIALS

Grass Management and Mowing Frequency. Six 15-cm-diam. cores of established stands of endophytic tall fescue, *F. arundinacea*, and endophytic perennial ryegrass, *L. perenne*, were taken from field plots established in 1999 at the Ohio Agricultural Research and Development Center. The cores were placed into 15-cm-diam. pots and transferred to a greenhouse in the fall of 2000. The

percentage of tillers infected by the endophyte was in the range of 30–100% with an average of 58% for the tall fescue, and 50–90% with an average of 77% for the perennial ryegrass (Grewal, unpublished data). Plants were maintained at 21°C day and 16°C night and a 16L:8D light cycle. Daylight was supplemented with artificial lighting as required. Plants were watered as a needed, and weekly applications of Peters Professional fertilizer [(20-20-20 N-P-K) (Scotts Company, Maysville, Ohio, USA] was provided. The plants were maintained in greenhouse for four weeks before sampling was initiated. The grasses were cut to 5-cm height either weekly or biweekly (every other week).

Sampling. Triplicate samples were collected monthly, starting one month after the first treatment. Sampling was limited as much as possible to a quarter section of the pot where the grass was cut at the soil level. We tried to avoid collecting from a previously sampled sector. Samples were immediately frozen in liquid nitrogen and lyophilized. Dried samples were ground to a powder in a Cyclotech 1093 mill and stored for analysis.

Alkaloid Extraction. A method described by Hill et al. (1993) with some modifications was used to extract alkaloids. Briefly, 100 mg of plant material were weighed and transferred into a liquid scintillation vial. One hundred nanograms of ergotamine were added as an internal standard. Nine ml of $CHCl_3$ and 1 ml of 0.01 N NaOH were added, and the sample was then agitated by wrist action for 30 min. The sample was filtered through Whatman 1 PS filter paper and loaded onto 5 ml solid-phase extraction columns prepared as described by Hill et al. (1993). The columns contained 0.5 g of Ergosil at the bottom and 1 g of Na_2SO_4 on top separated by Whatman No. 41 filter paper disks. Pigments were eluted with an aspirated manifold with 5 ml of acetone–chloroform (75:25), and the residual acetone–chloroform was removed with 1 ml of diethyl ether. A detachable Nylon 66 filter (22 μm) was attached to the syringe and the alkaloids eluted by slowly plunging 2 ml of methanol through. The sample was evaporated to dryness under N_2 flow and stored in the freezer untill analysis.

Separate extraction methods were attempted to see if they would be better suited for detection of peramine and lolitrem B in perennial ryegrass, as described by Ball et al. (1995). Only a few samples were subjected to these extractions. Chlorophylls remaining after the lolitrem extraction method were removed by passing the samples through an aminopropyl SPE column.

Data Collection. Samples were suspended in 100 μl of methanol, and analyzed on a Waters LC-MS system using a Novapak C_{18} (2 $\times$ 150 mm) column. The mobile phase was H_2O–methanol–acetonitrile plus 0.3% NH_4OH (60:10:20:10). The flow rate was 0.27 ml/min in a linear gradient for 48 min, changed to 10:10:70:10 at 52 min, and returned to the original composition untill 60 min. Peaks were detected with a Waters 996 Photodiode Array Detector, after which the flow was split in two with one part going through a Waters 474 Scanning Fluorescence Detector with excitation wavelength at 316 nm and emission

wavelength at 413 nm, and the other to the mass spectrometer. Splitting of the flow was done as a precautionary measure to avoid damage to the fluorometer cell by the pressure in the system. Positive ion electrospray spectra were recorded on a Waters ZQ instrument. Soft ionization of the electrospray results in production of quasimolecular ions $(M+H)^+$, where M represents molecular weight and H hydrogen.

Direct-phase chromatography of samples following Ball et al. (1995) was carried out as described by Gallagher et al. (1985) using a Supelco (2 × 150 mm) silica column and acetonitrile–CH_2Cl_2 (80:20) for 30 min. The excitation and emission wavelengths of the fluorescence detector were set at 265 nm and 440 nm, respectively.

Data Analysis. Quantification was based on the ergotamine internal standard. Repeated-measures (Statistica, StatSoft, Inc., Tulsa, Oklahoma, USA) analysis to look at the effect of time on alkaloid levels, and one-way ANOVA to compare samples from weekly and biweekly treatments at each sampling time were used. Replicates that could not be accurately determined due to interfering peaks in the chromatograms were excluded from analysis.

RESULTS

Alkaloids. Levels of major alkaloids and some of the unidentified putative alkaloids detected in tall fescue and perennial ryegrass are listed in Table 1. In tall fescue, we identified eight alkaloids, of which ergovaline, ergonovine, and ergocristine were the major ones. In addition, three unknown compounds were present in large quantities. In perennial ryegrass, ergocristine was the major alkaloid with two unidentified compounds. Evidence for eight more alkaloids was found using a method described by Ball et al. (1995).

Tall Fescue. The repeated-measures analysis of ergonovine indicated a significant effect by month (Figure. 1) ($F = 4.95$, $df = 3,12$, $P = 0.018$). Biweekly cutting resulted in higher levels of ergonovine ($F = 11.77$, $df = 1,3$, $P = 0.042$) by the second month.

The level of ergovaline in month 1 samples was higher in the grass subjected to weekly cutting than in grass clipped on a biweekly schedule ($F = 10.73$, $df = 1,3$, $P = 0.047$), but by month 2 the biweekly treatment showed a large increase over the weekly treatment ($F = 9.23$, $df = 1,4$, $P = 0.038$) (Figure 1). In the subsequent two months, a trend favoring biweekly mowing over weekly cutting was evident, but the differences were not significant due to large variation.

Like ergonovine, ergocristine levels showed an effect by month in the repeated-measures analysis ($F = 5.08$, $df = 3,12$, $P = 0.017$), but the cutting frequency was significant only at month 2 where weekly cutting gave higher values ($F = 6.81$, $df = 1,4$, $P = 0.059$). Levels remained high for the first three months and then decreased considerably in both treatments (Figure 1).

TABLE 1. ALKALOIDS AND PUTATIVE ALKALOIDS FOUND IN TALL FESCUE AND PERENNIAL RYEGRASS USING REVERSE-PHASE LC-MS ELECTROSPRAY IONIZATION[a]

Compound	$(M+H)^+$	Amounts (ng/g)
Tall fescue		
N-Formyl loline	183[b,c]	trace
N-Acetyl-loline	197	trace
Chanoclavine	257	<1
Ergonovine	326	71–490
Ergovaline	534	10–140
Ergocryptine	577	<1
Ergostine	596	trace
Ergocristine	611	84–312
Unknown A	537	370–4690
Unknown B	572	936–4196
Unknown C	574	48–391
Perennial ryegrass		
Peramine[b]	248	trace
Chanoclavine[b]	257	trace
Lysergamide[b]	268	trace
Lysergic acid[b]	269	trace
Ergonovine[b]	326	trace
Paxilline[b]	408	trace
Ergosine[b]	549	trace
Ergocristine	611	64–204
Lolitrem B[b]	686	trace
Unknown D	553	43–460
Unknown B	572	33–99

[a] The amounts (ng/g dry weight) are relative to an ergotamine internal standard. The compounds are in order of increasing size of their quasimolecular ions with known alkaloids listed first. $(M+H)^+$ denotes molecular weight (M) and hydrogen (H).

[b] Alkaloids found using the methods of Ball et al. (1995) and direct phase chromatography.

The three putative alkaloids (Figure 2, unknowns A, B, and C) all showed significant effects by month (A: $F = 63.02, df = 3,12; P = 0.001$; B: $F = 4.20, df = 3,12, P = 0.030$; C: $F = 3.66, df = 3,12, P = 0.044$), as well as month × treatment interactions (A: $F = 14.80, df = 3,12, P = 0.001$; B: $F = 8.34, df = 3,12, P = 0.003$; C: $F = 6.37, df = 3,12, P = 0.008$). During the second month of the study, all of the unknowns showed higher levels in samples from the biweekly cutting at month 2 (A: $F = 55.40, df = 1,4, P = 0.002$; B: $F = 103.27, df = 1,3, P = 0.002$; C: $F = 14.69, df = 1,4, P = 0.019$). Biweekly treatment also resulted in higher levels of unknowns A and B at month 4 (A: $F = 9.26, df = 1,4, P = 0.038$; B: $F = 9.59, df = 1,3, P = 0.053$).

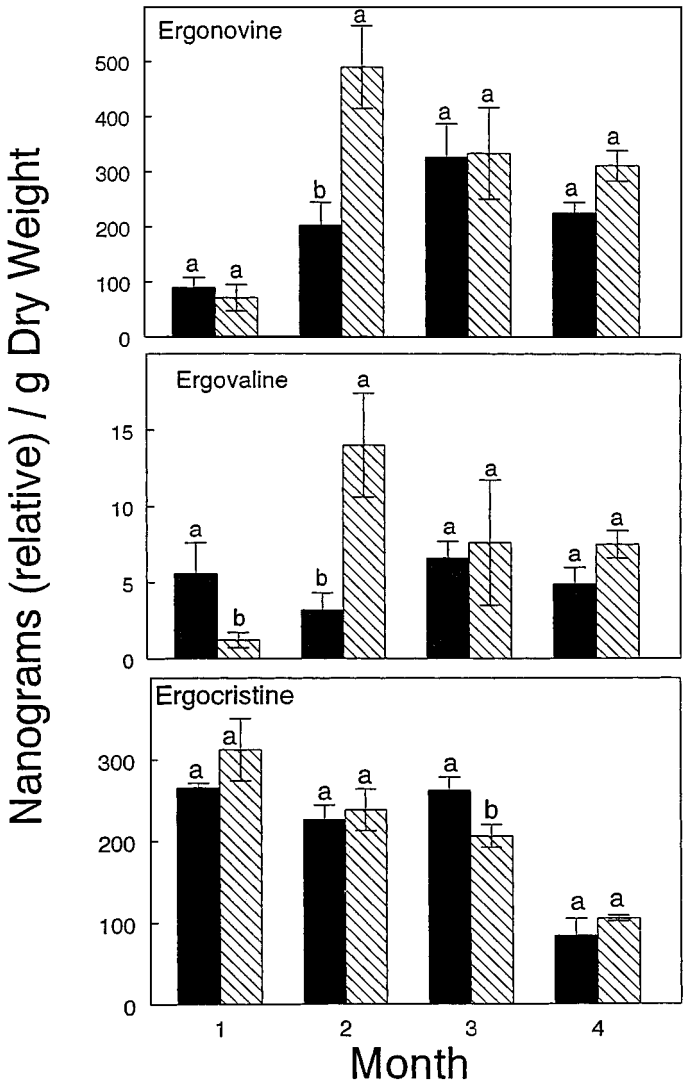


FIG. 1. Mean (±SE) levels of ergonovine, ergovaline, and ergocristine in tall fescue. Mowed weekly (■) or biweekly (▨). The same letters on the columns at each sampling date indicate no differences between treatments at $\alpha = 0.05$.

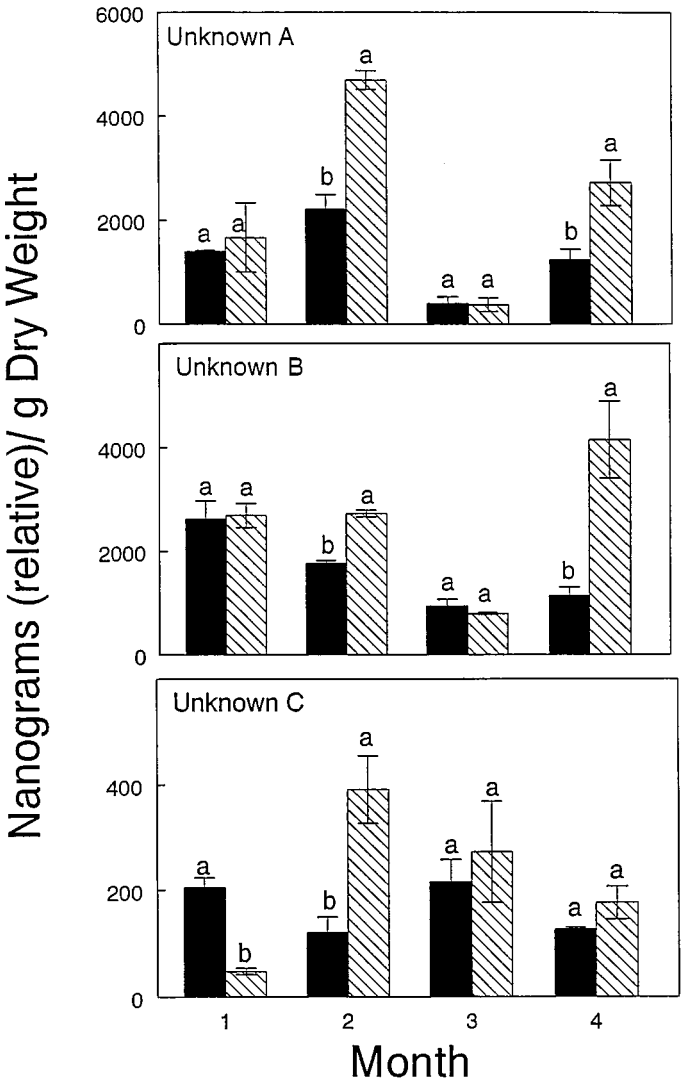


FIG. 2. Mean (±SE) levels of unknown A, unknown B, and unknown C in tall fescue. Mowed Weekly (■) or biweekly (▨). The same letters on the columns at each sampling date indicate no differences between treatments at $\alpha = 0.05$.

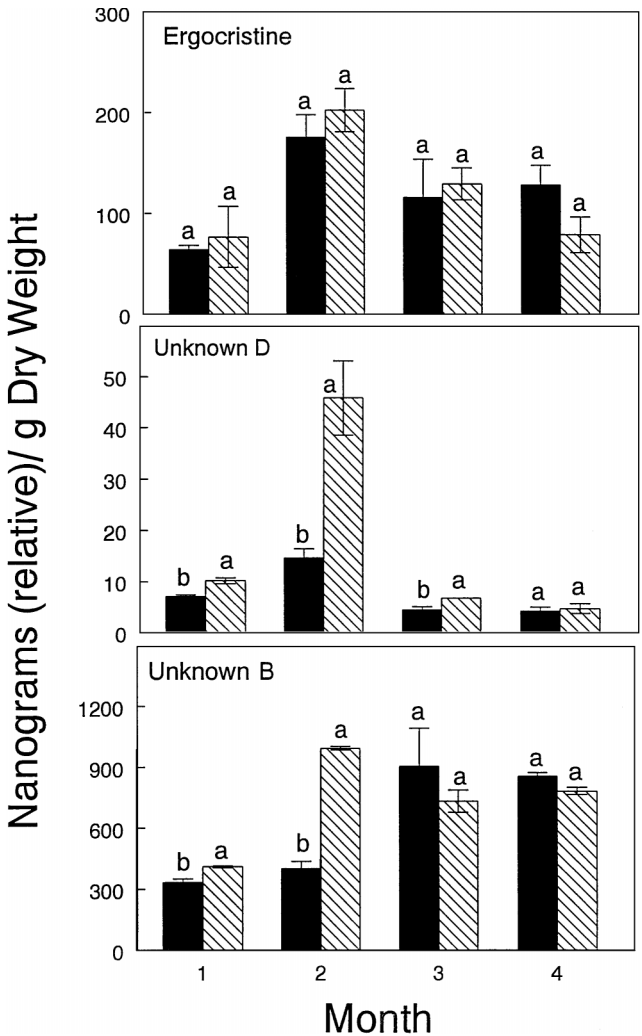


FIG. 3. Mean (±SE) levels of ergocristine, unknown D, and unknown B in perennial ryegrass. Mowed weekly (■) or biweekly (▨). The same letters on the columns at each sampling date indicate no differences between treatments at $\alpha = 0.05$.

Perennial Ryegrass. Only ergocristine was positively identified in the perennial ryegrass. Repeated-measures analysis indicated a main effect by month ($F = 8.17$, $df = 3,9$, $P = 0.006$), but no interaction. Biweekly treatments showed a slightly higher trend for the first three months over the weekly cutting (Figure 3). The level of ergocristine was similar to that in the tall fescue. For

unknown D, the repeated-measures analysis indicated a significant effect by month ($F = 8.17$, $df = 3,9$, $P = 0.005$) and month × treatment interaction ($F = 14.00$, $df = 3,3$, $P = 0.028$) (Figure 3). Levels of unknown D were higher in the biweekly treatment for the first three months (month 1: $F = 28.77$, $df = 1,3$, $P = 0.013$; month 2: $F = 17.17$, $df = 1,4$, $P = 0.014$; month 3: $F = 16.04$, $df = 1,2$, $P = 0.057$) (Figure 3).

Samples from biweekly treatments contained more of unknown B than those from the weekly mowing for the first two months (month 1: ($F = 18.14$, $df = 1,2$, $P = 0.051$; month 2: $F = 292.90$, $df = 1,2$, $P = 0.003$). Unknown B is the same compound as unknown B in the tall fescue based on the quasimolecular ion 572, its fragmentation pattern, and retention time in the chromatograph. In addition, traces of peramine, chanoclavine, and ergostine were observed in some of the samples, but they were either poorly resolved or not detected in others (data not shown). The identity of other unknown peaks remains to be elucidated. The presence of several known alkaloids was observed in the few samples extracted by using the method of Ball et al. (1997) (Table 1).

In weekly mowing treatments of both grasses, we observed an oscillating variation in the alkaloid levels. No evidence for this was seen in the biweekly mowing treatment.

DISCUSSION

Our results agree with those reported by others on the general composition of endophytic alkaloids in tall fescue and perennial ryegrass [(Porter et al., 1979; Lyons et al., 1986; Siegel et al., 1990)]. Ergovaline is generally considered to be the predominant alkaloid in tall fescue (Lyons et al., 1986). It was somewhat surprising that the levels of ergovaline that we found were low in comparison to the other identified alkaloids. However, the levels vary greatly depending on the endophyte–host combination (Siegel et al., 1990; Breen, 1993). Values as high as 8 mg/g dry weight for loline alkaloids in tall fescue have been reported (Jones et al., 1983). Ergovaline levels from 166 to 1083 ng/g dry wt plant tissue have been shown in tall fescue (Rottinghaus et al., 1991).

A common trend across all samples was the increased level of alkaloids in tall fescue samples subjected to biweekly mowing. Although differences in individual as well as in putative alkaloids were small, taken together they indicate a more consistent pattern. Small increases could have an important impact on herbivore performance if there were interactions between the alkaloids. Yates et al. (1989) reported synergistic interaction between perloline and ergocryptine on mortality of *Oniopeltus fasciatus* (Dallas). In fact, most clear-cut differences were seen at month 2. The exception to the trend was at month 1 where the weekly treatments sometimes had a higher alkaloid content than plants treated biweekly.

Alkaloids are more concentrated in the sheaths than in leaves. Rottinghaus et al. (1991) reported ergovaline levels in tall fescue up to three times higher in leaf sheaths than leaves. Our samples were not separated into sheaths and leaves, and the grass was cut to the soil level. Samples from weekly cuttings would be expected to have a higher sheath-to-leaf ratio. Had the data indicated higher alkaloid content in samples from weekly cutting, this would have reflected the sheath-to-leaf ratio. As our data indicate a higher level in biweekly treatments, this would argue that the differences would be even more pronounced if the sheath-to-leaf ratio were the same in the two treatments. This is consistent with the reduction of alkaloid levels as a result of clipping when clipped and nonclipped tall fescue were compared (Belesky and Hill, 1997). Belesky and Hill (1997) suggest that carbon from photosynthesis is used to build new leaf material, and is, thus, not available for alkaloid synthesis. This could also contribute to the low levels of ergovaline found.

The unknown compounds exhibited trends similar to the identified alkaloids. However, we cannot be certain that these were, in fact, alkaloids. The extraction method and the solid-phase extraction, although yielding alkaloids, do not necessarily exclude other compounds with similar properties, e.g., phenolic compounds and chlorophylls. It should be pointed out that the unknown with a pseudomolecular weight of 572 is not a $(M+K)^+$ adduct of ergovaline, based on the absence of the $(M+H)^+$ spectral ion at 534.

The methods used did not provide good data for peramine or lolitrem B in the perennial ryegrass. Preliminary results using methods developed for peramine and lolitrem B extraction and direct-phase chromatography gave indications of the presence of not only these compounds, but also of ergosine, perloline methyl ether, paxilline, lysergic acid, and lysergamide in the perennial ryegrass samples. Lysergamide (ergine) may arise from solvolytic cleavage of lysergic acid during the extraction (Agurell, 1966). These data suggest that it may be necessary to analyze samples using different extraction and chromatography procedures to get a more comprehensive picture of the alkaloid profile in these grasses, e.g., ethanolic extraction of turf grasses has been successfully used for analysis of ergosterol (Logendra and Richardson, 1997).

Results with the perennial ryegrass must be interpreted with some caution because of heavy infestation by the aphid *Rhopalosiphum padi.* Several species of aphids show no avoidance of alkaloids (Latch et al., 1985; Siegel et al., 1990). Tall fescue has been shown to be resistant to *R. padi* (Johnson et al., 1985) and was not attacked, although it was growing next to the infested perennial ryegrass. A difference in the composition of the alkaloids in the two grasses could be one explanation for this. Our efforts to control the aphids with judicious application of azidirachtin were moderately successful, as we tried to avoid possible complications arising from the use of a synthetic pesticide.

It is tempting to speculate that if the period between cuttings had been extended to three weeks, the differences might have been more pronounced. From the

standpoint of a homeowner or a golf course manager, however, that might be impractical. In many of the weekly treatments, we noticed oscillating fluctuations in the alkaloid levels. Oscillations usually result from negative feedback (Goldbeter and Dupont, 1990) and could indicate that synthesis of alkaloids is regulated by changes in metabolism resulting from cutting, as resources are allocated toward building of new leaf material at the expense of secondary metabolites. Oscillations were not seen in the biweekly treatment.

The results do suggest the possibility that management practices, such as mowing frequency, could have an impact on alkaloid levels, and, as a concequence, on insect pest control in turf grasses. Specific endophyte–host genotypes providing control over insect pests are becoming available. Replacing established lawns and golf course fairways might not always be economically feasible. However, overseeding is a viable alternative, provided the new seed can establish itself in an existing stand (Richmond et al., 2000). The inconsistent control of insects with the use of endophytes (Clay et al., 1985) may have its basis not only in the endophyte–host combination, but also in cultural practices such as mowing frequency.

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DIFFERENTIAL ATTRACTIVENESS OF INDUCED ODORS EMITTED BY EIGHT MAIZE VARIETIES FOR THE PARASITOID *Cotesia marginiventris*: IS QUALITY OR QUANTITY IMPORTANT?

MARIA ELENA FRITZSCHE HOBALLAH, CRISTINA TAMÒ, and TED C. J. TURLINGS*

Université de Neuchâtel, Institut de Zoologie
Laboratoire d'Ecologie Animale et Entomologie (LEAE)
Case Postale 2, 2007 Neuchâtel, Switzerland

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Abstract—Herbivore-induced plant volatiles can function as indirect defense signals that attract natural enemies of herbivores. Several parasitoids are known to exploit these plant-provided cues to locate their hosts. One such parasitoid is the generalist *Cotesia marginiventris*, which is, among others, attracted to maize volatiles induced by caterpillar damage. Maize plants can be induced to produce the same blend of attractive volatiles by treating them with regurgitant of *Spodoptera* species. We collected and analyzed the regurgitant-induced emissions of two plant species (cowpea and maize) and of eight Mexican maize varieties and found significant differences among their volatile emissions, both in terms of total quantity and the quality of the blends. In a Y-tube olfactometer, the odors of the same artificially induced plant species and Mexican varieties were offered in dual choice experiments to naïve mated females of *C. marginiventris*. Wasps preferred cowpea over maize odor and, in 3 of 12 combinations with the maize varieties, they showed a preference for the odors of one of the varieties. A comparison of the odor collection with results from the behavioral assays indicates that not only the quantity of the volatile emissions, but also the quality (composition) of the volatile blends is important for attraction of *C. marginiventris*. The results are discussed in the context of the possibility of breeding crop varieties that are particularly attractive to parasitoids.

Key Words—Induced plant volatiles, parasitoid, olfactometer, *Cotesia marginiventris, Spodoptera littoralis*, *Zea mays*, *Vigna unguiculata*, parasitoid attraction, odor variability.

* To whom correspondence should be addressed. E-mail: ted.turlings@unine.ch

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INTRODUCTION

Herbivore-induced odors have been suggested to benefit plants in different ways (Vet and Dicke, 1992; Turlings and Benrey, 1998; Sabelis et al., 1999; Dicke and van Loon, 2000). Over the last 15 years, it has become evident that these odors are important for the attraction of natural enemies to the microhabitat of their prey (e.g., Dicke et al., 1990a; Turlings et al., 1990a; Steinberg et al., 1993; Du et al., 1998; Röse et al., 1998). The blend of odors released by plants after herbivore attack is variable, depending on the plant (species and variety) and the herbivore that feeds on it, although some compounds are common to many induced blends (for reviews see Dicke, 1994, 1999). It is known that European maize varieties and their wild ancestors differ considerably in quality and quantity of odors if induced to produce volatiles by treating scratched leaves with *Spodoptera littoralis* (Lepidoptera: Noctuidae) regurgitant (Gouinguené et al., 2001). We define differences in the quality of the odor blends as differences in the presence of specific compounds in the blend and/or the proportions among compounds. This variation among maize varieties could have important consequences for pest management, as biological control may be more effective for varieties that are highly attractive to natural enemies. This may be particularly true for countries in Latin America where the major pests of maize are leaf-feeding *Spodoptera* caterpillars (for review see Andrews, 1988).

Young *Spodoptera* caterpillars can be parasitized by *Cotesia marginiventris* (Hymenoptera: Braconidae), a common solitary endoparasitoid that attacks Lepidopteran larvae during the first and second instar (Jalali et al., 1987). *C. marginiventris* females are strongly attracted by the odors emitted by caterpillar-damaged maize seedlings (Turlings et al., 1990a, 1991a) and spend more time on caterpillar-damaged plants than on artificially damaged or unattacked plants (Loke et al., 1983). Maize plants infested with a *Spodoptera* larva parasitized by *C. marginiventris* produce more seeds than plants infested with a healthy caterpillar (Fritzsche Hoballah and Turlings, 2001), which could translate into a higher yield for maize cultivars that are particularly attractive to the parasitoid. Hence, it seems important to know which factors determine the attractiveness of the crop.

Here we report on how differences in induced volatile emissions among different plant genotypes may affect the attractiveness of these genotypes to parasitoids. We first compared the emissions of maize and cowpea and their attractiveness to *C. marginiventris*. The former plant species is known to release large amounts of induced volatiles (e.g., Turlings et al., 1998), while the latter releases relatively little, but can be quite attractive (Whitman and Eller, 1990). Furthermore, we investigated the attractiveness of eight Mexican maize cultivars. In Y-tube olfactometer, naïve *C. marginiventris* females were offered a choice between the odors of the two respective plant species or 12 paired combinations of the Mexican maize varieties. Comparison of the results from these behavioral assays with the

odor blends that were collected from the various plants revealed that for naïve wasps attractiveness is not simply a matter of quantity of volatiles, but that the quality of the blend is important as well.

METHODS AND MATERIALS

Growing and Treatment of Plants. Plants used in all experiments were grown in plastic pots (6 cm high, 8 cm diam.) in fertilized commercial soil (Coop) in a climate chamber (23°C, 60% relative humidity, and 16L:8D, 50,000 lumens/m^2). Two-week-old *Vigna unguiculata* (var. kpodii-guegue, obtained from IITA) Africa) and 10-day old *Zea mays* (var. Delprim and varieties obtained from CIMMYT, Table 1) plants were used for the experiments. To induce the plants to produce volatiles, we scratched two leaves (the second and third leaf of maize and first and second of cowpea) of each plant with a razor blade (an area of 2–4 cm^2/leaf) and subsequently applied on each damaged site 10 μl of regurgitant. Regurgitant was collected from fourth and fifth instar *S. littoralis* caterpillars (for the method, see Turlings et al., 1993a) and kept at −70°C until just before utilization. After treatment, plants were placed for 5 hr (plant species) and 6 hr (varieties), under three fluorescent lamps (Sylvania standard F36W 133-T8 cool white, 5000 lumens/m^2 at pot height), before they were used for the volatile collection and olfactometer experiments. It is known that 5–6 hr after treatment corn plants emit relatively large quantities of induced volatiles (Turlings et al., 1998).

Insects. *S. littoralis* caterpillars and eggs were supplied weekly by Syngenta (Stein, Switzerland). Eggs were incubated in Petri dishes (9 cm diam., 1.5 cm high) on moist filter paper. Emerged caterpillars were fed with artificial diet and kept in plastic-boxes (15 × 9 × 5 cm) under ambient laboratory conditions. This non-native host causes a similar reaction in plants as the natural host *S. frugiperda* (Fritzsche Hoballah, unpublished data).

TABLE 1. VARIETY NUMBER, NAME, ORIGIN, AND SEED COLOR OF 8 MEXICAN CORN VARIETIES USED FOR VOLATILE COLLECTION AND CHOICE EXPERIMENTS

Variety	Name	Origin, seeds color
1	Its 2 Sint. A1# Bulk	PR-99-A 761-A yellow
2	Its 2 Sint. A1# Bulk	PR-99-A 761-B white
3	Its 2 Sint. B3# Bulk	PR-99-A 761-B white
4	Its 2 Sint. A1# Bulk	PR-98-B 5766-A yellow
5	Its 2 Sint. B1# Bulk	PR-98-B 5766-B white
6	P36 C9 HC60-B-1-BBBBBBBBB-2 CL-03618	PR-99 A 402-16 yellow
7	CML287(P24F26*P27F1)-4-1-B-1-1-BB-F-####-BBB	PR-98 A 479-11 yellow
8	CL-00331SINT.AM.TSR-23-3-2-3-2-BB-F-##-B-B CML413	PR-98 A 474-16 yellow

The solitary endoparasitoid *C. marginiventris* that was used in the experiments originated from the USDA-ARS, Biological Control and Mass Rearing Research Unit. For the rearing of parasitoids, 25 young caterpillars (3–4 days old) were offered to a single mated female (4–7 days old) for 3 hr in a plastic box (9.5 cm diam., 5 cm high). The caterpillars were further reared on artificial diet in an incubator (25°C and 16L:8D) until cocoon formation. Cocoons were kept in Petri dishes until adult emergence. Emerging adults were sexed and kept in cages (30 × 30 × 30 cm) at a male–female ratio of 1:2, with distilled water on cotton and honey as a food source. The cages were kept in the laboratory under ambient light and temperature conditions.

Volatile Collection. After treatment and light exposure, plants were placed in a climate chamber (CMP4030, Conviron, Winnipeg, Canada) and the emitted volatiles were collected. The climate chamber was programmed at 20°C and with a light intensity of 14,500 lumens/m^2 (light provided by 28 lamps, 16 Sylvania 150-W VHO Cool White and 12 Sylvania 100-W Satin). Volatiles were collected from six plants simultaneously in an automated collection system as described by Heath and Manukian (1992) and Turlings et al. (1998). Glass cylinders (9.5 cm diam., 54 cm high) were placed over the plants and rested on a Teflon disk consisting of two halves with a hole in the middle for the stem of the plant. Air was pushed (1 liter/min) through a charcoal filter and introduced from the top of each cylinder over the plants. Super-Q traps (25 mg, 80/100 mesh, Alltech, Deerfield State) were attached laterally at the base of each cylinder. Traps were connected to an automated volatile collection system (ASU, ARS, Gainesville, Florida) through which the air was pulled out at 0.8 liters/min during 2 hr. The collection period was programmed with the use of the software TESS (Version 1.0, ARS, Gainesville, Florida). After each collection, volatiles were extracted from the traps with 150 μl methylene chloride, and 200 ng of *n*-octane and nonyl acetate were added as internal standards. Aliquots (3 μl) of the samples were injected on column, with an automated injection system into a Hewlett Packard model HP 6890 gas chromatograph equipped with a flame ionization detector. The apolar EC-1 capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness, Alltech Associates) was held at 50°C for 3 min and then programmed at 8°C per min to 230°C, where it was maintained for 9.5 min. The column was preceded by a deactivated retention gap (10 m × 0.25 mm ID, Connex) and a deactivated precolumn (30 cm × 0.530 mm ID, Connex). Helium (24 cm/sec) was used as carrier gas. We collected and analyzed the odor of treated cowpea and maize plants ($N = 6$) as well as of 8 Mexican varieties ($N = 5$ for each variety). Hewlett Packard GC Chemstation software was used to quantify all major components by comparison to the known quantity of internal standards. Initial identification of most compounds was based on comparisons of retention times from previous studies (Turlings et al., 1998; Bernasconi et al., 1998). Conformation of identity by comparison of retention times with the synthetic samples was obtained for nerolidol (Fluka), (*E*)-β-farnesene (Denka

International), geranyl acetate (Fluka), (*Z*)-3-hexenol (Fluka), (*Z*)-3-hexenyl acetate (Sigma), linalool (Fluka), indole (Fluka), and β-myrcene (Sigma). A few compounds were identified only with the use of the Wiley library after mass spectrometry analysis (Agilent 5973, transfer line 230°C, source 230°C, quadrupole 150°C, ionization potential 70 eV, scan range 0–400 amu). These compounds are 2-hexenol, β-sesquiphellandrene, β-bisabolene, and cycloisosativene/α-ylangene. These identifications are regarded as tentative and are marked as such in the remainder of the text.

ANOVA and Student-Newman-Keuls *post-hoc* test were used to compare the total quantity of volatiles emitted among varieties (data ln-transformed). The Mann-Whitney test was used to compare differences in total emissions between plant species and among single compounds emitted by the different species and plant varieties. Tests were carried out with the program SPSS 10.0 for Windows.

Choice Experiments. A Y-shaped olfactometer was used to test the attractiveness of plant odors to *C. marginiventris* females. The system, based on a design by Sabelis and Van de Baan (1983), consisted of a central tube (13.5 cm long, 4 cm diam.) and two lateral arms (12 cm long, 4 cm diam.) ending with a glass frit (to create a homogeneous airflow and to prevent escape of test insects). The lateral arms tapered off into 0.5-cm tubes to which Teflon tubing was connected. Teflon tubes were used to introduce the odors into the Y-tube. To limit visual distractions for the insects, the Y-tube olfactometer was placed inside a white paper box, which was open on top (for illumination) and on the front side (for observation). An office lamp (60 W, 750 lumens/m^2) illuminated the olfactometer. A black and white striped pattern for visual orientation was placed under the Y-tube. Plants, treated and incubated in the same way as for volatile collections, were placed in plastic bags that were then heat-sealed. Humidified and purified (with activated charcoal) air was pushed into each bag via Teflon tubing and airtight connectors at pot height. The air passed over the plants and into the Teflon tubes connected with the arms of the olfactometer at a rate of 0.8 liters/min.

Wasps were used only one time and released singly at the base of the central arm of the Y-tube. In a first experiment, they were offered the odor of induced cowpea and maize (var. Delprim). In subsequent experiments, they had the choice between the odor of two different induced Mexican maize varieties. Twelve of the 36 possible combinations of the eight Mexican varieties were randomly chosen for these tests. It was not our goal to determine which of the eight varieties were the most attractive, but to establish if only the quantity or also the quality of the blends were important for the attraction of *C. marginiventris*. Naïve (no experience with hosts or plants) wasps were placed individually in the base of the olfactometer and observed for 10 min. If a wasp did not make a choice after this period, it was removed and recorded as a "no choice." Wasps that walked to the end of one of the arms and stayed there at least 5 secs were recorded as having made a choice for the odor offered through that arm. After four to seven individuals were tested,

the olfactometer was turned such that the direction of the arms was reversed and another four to seven wasps were tested. Each combination of odor sources was tested six to nine times with different plants and on different days. Each time the position of the two different odor sources was switched. A minimum of 67 and maximum of 94 wasps were tested in the 12 combinations of maize odor sources.

A binomial test was used to determine significant preferences for an odor. The number of wasps that chose the highest and lowest volatile emitting maize varieties on each experimental day was compared with a Wilcoxon signed rank test. This test was carried out using data of all experimental days to determine if the quantity (regardless of quality) of the odor was important for the attraction of the wasps.

RESULTS

Volatile Collection. The two plant species, cowpea and maize, differed dramatically in the quantities of volatiles emitted (Figure 1). The total amount of volatiles collected during the experiment was almost 53 times higher in maize (4141.35 ± 460.2 ng/2 hr) than in cowpea (77.9 ± 20.9 ng/2 hr) ($N = 6$, Mann-Whitney test: $P = 0.004$, $Z = -2.882$). The two plant species released several identical compounds, but cowpea did not release benzyl acetate, phenethyl acetate, 1-H-indole, (*E*)-β-caryophyllene, "β-sesquiphellandrene," or (*E*, *E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and it released only traces of (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, α-(*E*)-bergamotene, (*E*)-β-farnesene, "β-bisabolene," and nerolidol (Figure 1). In contrast, cowpea emitted considerable amounts of (*E*)-2-hexenal and significantly more (*Z*)-3-hexenol than maize plants (Figure 1).

Among the Mexican maize varieties we also found differences in the total amounts of volatiles collected ($N = 5$, ANOVA: $df = 7$, $F = 10.589$, $P < 0.001$, Figure 2). Variety 1 emitted 14 times more than variety 8 (Figure 2). We compared the emissions of volatiles for the varieties that were shown to be differentially attractive to *C. marginiventris* (see below). Variety 4 released significantly more (*Z*)-3-hexenal, (*Z*)-3-hexenol, and phenethyl acetate than variety 7 (Figure 3A). Variety 4 did not release geranyl acetate or (*E*)-β-ocimene, and variety 7 did not release "cycloisosativene/α-ylangene" or "β-sesquiphellandrene" (Figure 3A). Variety 1 released significantly more (*Z*)-3-hexenyl acetate, (*E*)-4,8-dimethyl-1,3,7-nonatriene, benzyl acetate, phenethyl acetate, geranyl acetate, and "cycloisosativene/α-ylangene" than variety 6 (Figure 3B). Variety 6 did not release (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, benzyl acetate, or phenethyl acetate (Figure 3B). Variety 1 released significantly more linalool, benzyl acetate, phenethyl acetate, 1-H-indole, geranyl acetate, α-(*E*)-bergamotene, (*E*)-β-farnesene, and "β-sesquiphellandrene" than variety 5 (Figure 3C). Variety 5 did not produce "cycloisosativene/α-ylangene" (Figure 3C).

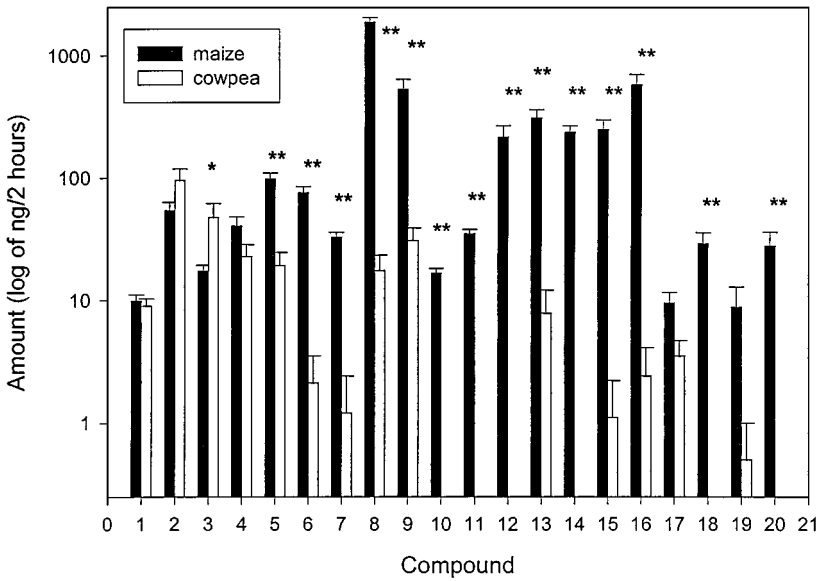


FIG. 1. Mean amount (±SE) of volatiles collected from cowpea and maize plants that were artificially damaged and treated with regurgitant of *Spodoptera littoralis* caterpillars. Collections started 5 hr after treatment and lasted 2 hr. Asterisks above bars indicate significant differences (Mann-Whitney test (two-tailed): $^{*}P < 0.05$, $^{**}P < 0.005$) in release of a specific compounds. The compounds are: **1**, 1-(*Z*)-3-hexenal; **2**, (*E*)-2-hexenal; **3**, (*Z*)-3-hexenol; **4**,"2–hexenol"; **5**, β-myrcene; **6**, (*Z*)-3-hexenyl acetate; **7**, (*E*)-β-ocimene; **8**, linalool; **9**, (*E*)-4,8-dimethyl-1,3,7-nonatriene; **10**, benzyl acetate; **11**, phenethyl acetate; **12**, 1-H-indole; **13**, geranyl acetate; **14**, (*E*)-β-caryophyllene; **15**, (*E*)-α-bergamotene; **16**, (*E*)-β-farnesene; **17**, "β-bisabolene"; **18**, "β-sesquiphellandrene"; **19**, nerolidol; **20**, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (compounds within quotation marks were tentatively identified by comparison of their MS-spectra with those of the Wiley library).

Choice Experiments. Cowpea odors were more attractive to *Cotesia marginiventris* than maize odors (Binomial test, $P = 0.007$, Figure 4). The parasitoids showed only a preference in 3 of the 12 combinations of Mexican maize varieties tested (Figure 4). In one case (var. 1 vs. var. 5), the wasps were attracted to the variety releasing more volatiles (Figures 2, 3C, and 4). However, in two cases (var. 4 vs. var. 7 and var. 1 vs. var. 6), the varieties tested released the same amounts of volatiles (Figures 2, 3A,B, and 4), and the preference must have been due to a difference in odor quality.

The number of wasps on each experimental day (89 experimental days in total) choosing the higher emitting maize variety on that day (mean ± SE wasps, 4.6 ± 0.25) was compared with the number of wasps on each experimental day choosing

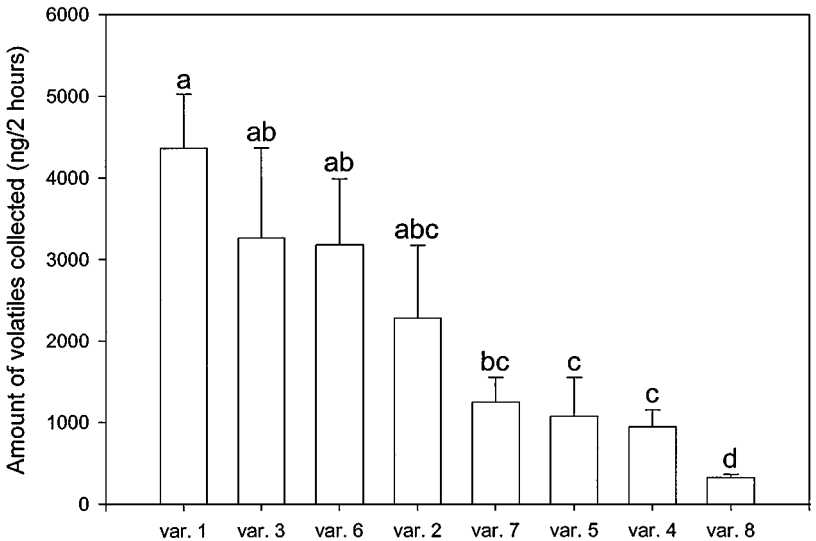
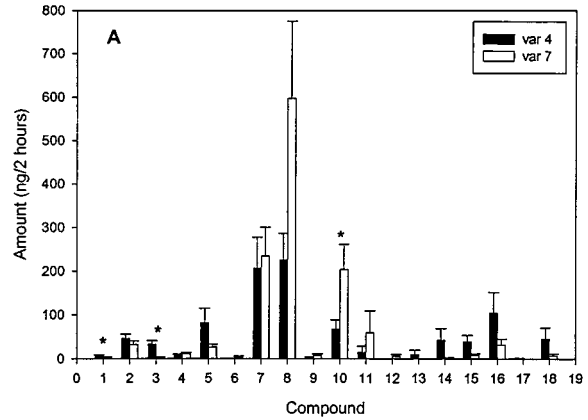
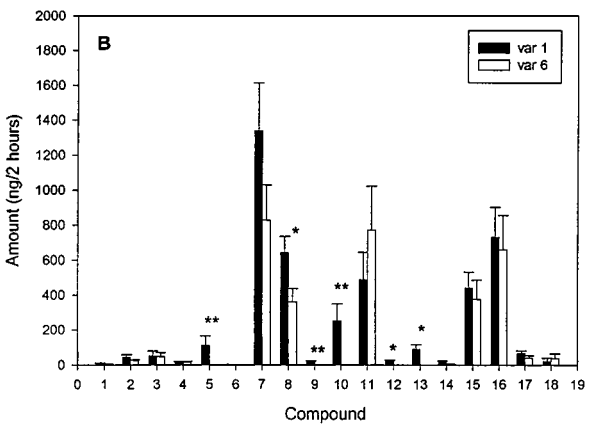


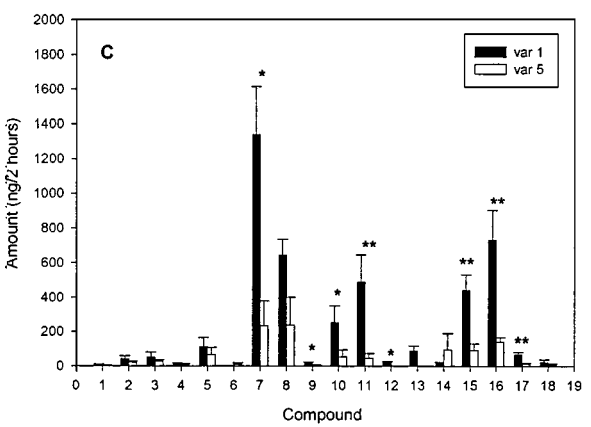
FIG. 2. Mean amount (±SE) of volatiles collected from different varieties of maize seedlings (10 days old) that were artificially damaged and treated with regurgitant of *Spodoptera littoralis* caterpillars. Volatiles were collected 6 hr after treatment, for 2 hr. Letters above bars indicate significant differences between varieties in the total amount released (Student-Newman-Keuls post hoc test after ANOVA: $df = 7$, $F = 10.589$, $P < 0.001$).

the lower emitting maize variety (3.8 ± 0.24 wasps). It revealed a marginally significant preference for the variety that emits larger amounts of volatiles offered in the dual choice test (Wilcoxon signed rank test: $P = 0.045$, $Z = -2.008$).

FIG. 3. Mean amount (±SE) of individual compounds collected from maize varieties that were artificially damaged and treated with regurgitant of *Spodoptera littoralis* caterpillars. Collections started 6 hr after treatment and lasted 2 hr. Asterisks above bars indicate significant differences (Mann-Whitney test (two-tailed): $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$), between the two plant varieties (A: between varieties 4 and 7, B: 1 and 6, and C: 1 and 5). The compounds are: **1**, 1-(*Z*)-3-hexenal; **2**, (*E*)-2-hexenal; **3**, (*Z*)-3-hexenol; **4**, β-myrcene; **5**, (*Z*)-3-hexenyl acetate; **6**, (*E*)-β-ocimene; **7**, linalool; **8**, (*E*)-4,8-dimethyl-1,3,7-nonatriene; **9**, benzyl acetate; **10**, phenethyl acetate; **11**, 1-H-indole; **12**, geranyl acetate; **13**, "cycloisosativene/α-ylangene"; **14**, (*E*)-β-caryophyllene; **15**, (*E*)-α-bergamotene; **16**, (*E*)-β-farnesene; **17**, "β-sesquiphellandrene"; **18**, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (compounds within quotation marks were tentatively identified by comparison of their MS-spectra with those of the Wiley library).


A
var 4
var 7
Amount (ng/2 hours)
Compound


B
var 1
var 6
Amount (ng/2 hours)
Compound


C
var 1
var 5
Amount (ng/2 hours)
Compound

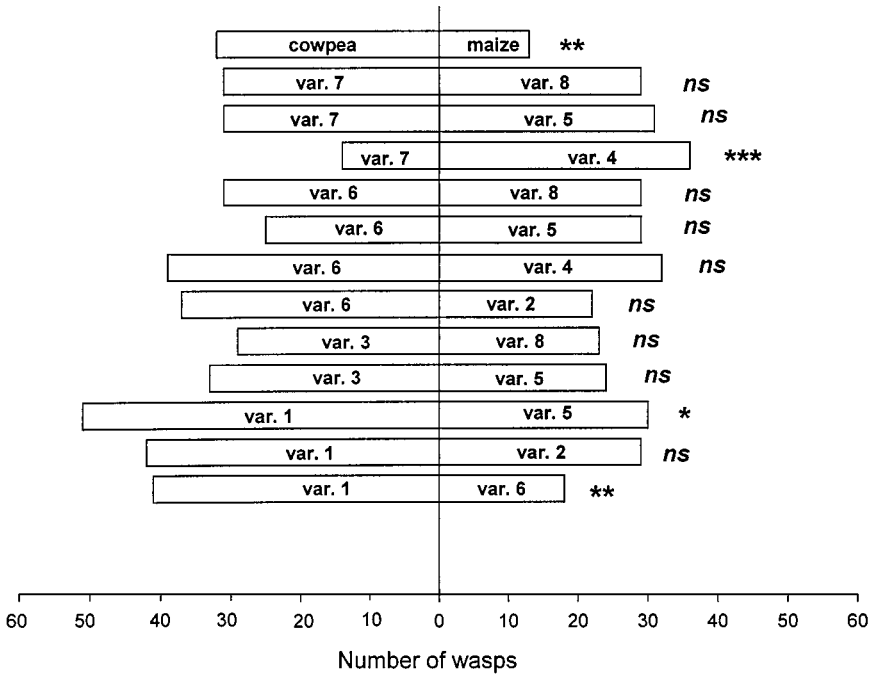


FIG. 4. Number of choices made by naïve *Cotesia marginiventris* in dual choice tests with various plant combinations offered in a Y-tube olfactometer. Asterisks indicate a significant difference within a choice test ($^{*}P < 0.01$, $^{**}P < 0.001$, $^{***}P < 0.0001$ and ***ns*** indicates no significant difference (binomial test).

DISCUSSION

Difference in Induced Volatile Emission among Plant Species and Varieties. Differences in herbivore-induced odor emissions among plant species genotypes have been demonstrated in several studies (Takabayashi et al., 1991, 1994a; Loughrin et al., 1995; Geervliet et al., 1997; Halitschke et al., 2000; Ozawa et al., 2000). In maize, these differences can be considerable (Gouinguené, et al., 2001). The current study confirms that different plant species, but also different varieties, release qualitatively and quantitatively different blends of induced odors. The 11 most important compounds that were collected from induced maize plants are partially shared with other plant species for which induced volatiles have been studied (Table 2). The only compound released by all plant species is (*Z*)-3-hexenol, and in 12 of 14 cases, (*Z*)-3-hexenyl acetate and (*E*)-4,8-dimethyl-1,3,7-nonatriene are also shared (Table 2).

TABLE 2. VOLATILES COLLECTED FROM REGURGITANT-INDUCED MAIZE PLANTS, SHARED AMONG OTHER PLANT SPECIES[a]

Compound	Apple	Bean	Brussels sprouts	Cabbage	Cucumber	Cotton	Gerbera	Pear	Potato	Soybean	Tobacco	Cowpea
1-(*Z*)-3-hexenal						+				+		+
(*E*)-2-hexenal	+(*E*?)	+	+	+		+		+		+		+
(*Z*)-3-hexenol	+	+	+	+	+	+	+	+	+	+	+	+
2-hexenol	+		+			+		+				+
β-myrcene	+(β?)		+	+(β?)		+(β?)	+(β?)					+
(*Z*)-3-hexenyl acetate	+	+	+	+	+	+	+	+		+		+
(*E*)-β-ocimene	+	+			+	+	+(*Z*)	+			+	+
linalool	+	+		+			+	+	+		+	+
(*E*)-4,8-dimethyl-1,3,7-nonatriene	+	+	+	+	+	+	+(*Z*)	+	+			+
benzyl acetate												
phenethyl acetate												
1-H-indole				+		+	+			+		
geranyl acetate												+
(*E*)-β-caryophyllene	+(*E*?)					+	+	+(*E*?)	+		+	
(*E*)-α-bergamotene							+		+		+(*Z*)	+
(*E*)-β-farnesene	+(*E*?)		+(α-)		+((*E*,*E*)-α)	+(γ-)	+	+(α)	+		+((*E*,*E*)-α)	+
β-bisabolene							+		+			+
β-sesquiphellandrene							+		+			
nerolidol							+					+
(*E*,*E*)-4,8,12-trimethyl 1,3,7,11-tridecatetraene	+	+	+	+	+	+	+(di-)		+			

[a] References: apple: Takabayashi et al. (1991, 1994a); lima bean: Dicke et al. (1990b); brussels sprouts: Mattiacci et al. (1994); cabbage: Agelopoulos and Keller (1994), Blaakmeer et al. (1994), Geervliet et al. (1997); cucumber: Dicke et al. (1990b), Takabayashi et al. (1994b); cotton: McAuslane and Alborn (1998), Loughrin et al. (1995), McCall et al. (1994); gerbera: Gols et al. (1999); pear: Scutareanu et al. (1997); potato: Weissbecker et al. (2000), Agelopoulus et al. (2000), Bolter et al. (1997); soybean: Turlings et al. (1993b); tobacco: De Moraes et al. (1998), Halitschke et al. (2000); cowpea: present study.

We found large qualitative and quantitative differences in induced odor blends between the plant species, maize and cowpea, and to a lesser extent among different maize varieties. Cowpea released almost exclusively green leaf volatiles, as was found by Whitman and Eller (1990), and maize released more indole, linalool, and a range of terpenoids that differed in the presence, proportion, and amount among varieties. Among maize varieties, the difference in total emission of volatiles was striking, with a 14-fold difference between lowest and highest emitting variety. When comparing the odors released by plants infested by *Pieris* larvae, Geervliet et al. (1997) showed differences in odor blends released by different plant species and cabbage varieties. Takabayashi et al. (1991, 1994a) found a qualitative and quantitative difference in volatile compounds emitted between two varieties of apple attacked by the same spider mite. In a comparison of *Spodoptera exigua*-infested leaves from different cotton varieties, naturalized cotton released seven times more volatiles than commercial cultivars (Loughrin et al., 1995). One constraint of using insects to induce odor emissions is that observed differences could be due simply to the fact that herbivores eat more or less of a specific plant species or variety. Using elicitors may allow for a more precise comparison. This was done by Halitschke et al. (2000), who treated the leaves of three genotypes of *Nicotiana attenuata* with methyl jasmonate and found a difference in the pattern of emitted compounds. The standardized treatment of the Mexican maize varieties in our study rules out any variable treatment effects. The results corroborate the tremendous variability in induced odor emissions in the genus *Zea* (see Turlings et al., 1998; Gouinguené et al., 2001).

Responses of Natural Enemies to Plant Odors. It is known that parasitoids show innate preferences to odors of specific undamaged plant species (Ngi-Song et al., 1996; Vaughn et al., 1996; Benrey et al., 1997). Furthermore, parasitoids show innate preferences for odors of specific plant varieties. For example, the parasitoid *Campoletis sonorensis* was attracted differentially to odors released by different cotton varieties (Elzen et al., 1986). In contrast, the parasitoid *Diaeretiella rapae*, did not distinguish among six grass varieties presented in a flight tunnel assay (Vaughn et al., 1996). If plants are damaged by herbivores, odors subsequently produced and released by the plants are considerably more attractive to parasitoids than odors of undamaged plants (Turlings et al., 1991b; Steinberg et al., 1993; Blaakmeer et al., 1994; Agelopoulos et al., 1995; Finidori-Logli et al., 1996; Bertschy et al., 1997; Cortesero et al., 1997; Röse et al., 1998). Choice experiments carried out in flight tunnels and in semi-field plots show that parasitoids have preferences for specific herbivore-attacked plant species (Drost et al., 1988; Geervliet et al., 1996; Du et al., 1998; De Moraes and Lewis, 1999).

Our study compared the attraction of *C. marginiventris* females to cowpea and maize and among maize varieties, focusing exclusively on odor cues. Cowpea odors were preferred over maize odors by naïve *C. marginiventris* females. This was somewhat surprising because cowpea released much less than maize. We had

expected the quantity of odors to be positively correlated with attractiveness, as is indicated by dose-dependent responses of natural enemies in several studies (Eller et al., 1988; Turlings et al., 1990a, 1991b; Ngi-Song et al., 1996; Vaughn et al., 1996; Weissbecker et al., 1999). Although the dual-choice experiments with maize varieties showed that, in general, *C. marginiventris* is more attracted to high releasing maize varieties, in a few combinations females showed preferences even if the quantities of odors produced by the two varieties did not differ. These results indicate that some compounds are more important than others for attraction of wasps, and that the proportions among compounds in the blend could play an important role. Cowpea released relatively larger proportions of green leaf volatiles as (*Z*)-3-hexenol, a very common compound among plant-induced volatiles (Table 2), than maize. Cortesero et al. (1997) analyzed the responses of *C. marginiventris* females to cotton plants and suggested that they primarily cue in on recently damaged plants, which are known to release green leaf volatiles. Other parasitoids like *Microplitis croceipes* and *Netelia heroica* are also attracted to green leaf volatiles, especially to different hexenols and hexenals, when they are offered individually in a flight tunnel (Whitman and Eller, 1990). The parasitoid *Aphidius rhopalosiphi* was strongly attracted to the two green leaf volatiles (*Z*)-3-hexenyl acetate and (*E*)-2-hexenal in Y-tube experiments (Wickremasinghe and Van Emden, 1992). These same chemicals are involved in the attraction of *C. glomerata* to infested or regurgitant-treated cabbage plants (Mattiacci et al., 1994). Green leaf volatiles are probably the most common volatiles released by plants damaged by herbivores (Table 2), which could explain why natural enemies without previous experience (naïve) are strongly attracted to them. (*Z*)-3-hexenol, which is shared by all plant species listed in Table 2, elicits strong responses in the two-spotted stinkbug predator *Perillus bioculatus*, while β-caryophyllene, a terpenoid shared by half of the plant species, elicited only weak responses (Weissbecker et al., 1999). Yet, the importance of specific plant volatiles and mixtures of volatiles has been suggested for the attraction of some natural enemies. The lacewing *Chrysopa carnea*, a predator of small insects, was caught more often in traps containing β-caryophyllene, than in traps that contained limonene, bisabolene, or caryophyllene oxide (Flint et al., 1979). In contrast, β-caryophyllene did not elicit antenna palpation by the parasitoid *Campoletis sonorensis*, whereas caryophyllene oxide did (Elzen et al., 1984). The EAG response of the two spotted stinkbug *Perillus bioculatus* was strong for a α-zingiberene and bicyclogermacrene mixture, but low/when these two compounds were offered singly to the predator (Weissbecker et al., 2000). The volatile compound borneol, extracted from garry oak, *Quercus garryana*, attracted the tachinid parasitoid *Cyzenis albicans* (Roland et al., 1995).

Innate Versus Learned Responses. Generally, naïve females respond poorly to odor cues, but a contact experience with hosts and/or host feces increases this responsiveness (Dmoch et al., 1985; Wardle and Borden, 1989; Whitman and Eller, 1990; Steinberg et al., 1992). These experiences can increase the insects'

motivation to search for a host, but this is also the result of associative learning of the encountered odors (Turlings et al., 1990b, 1993b; Vet and Groenewold, 1990). *C. marginiventris* females also show a dramatic increase in responses to herbivore-damaged maize plants in a flight tunnel after the females have contacted a host-damaged plant (Turlings et al., 1989, 1990b, 1993b). The olfactometer assays allowed us to better test odor preferences in naïve females. The strong responses to cowpea indicate that the innate preference is for the most common herbivore-attacked plant volatiles, the green leaf volatiles. Once a host is encountered, typical host-induced volatiles, like specific terpenoids, may be associated with host-presence, and the wasps will use these more reliable cues in searching for more hosts (Vet and Dicke, 1992). The fact that naïve females are less responsive to specific odor cues could explain why we found only three clear preferences among the 12 combinations tested in the Y-tube olfactometer. Nevertheless, the results suggest that *C. marginiventris* has some innate preference for certain odor blends. To reveal the key compounds in these blends, it would be useful to test the response of the parasitoids to plants that differ only in a single compound as suggested by Dicke and van Loon (2000).

Selection of Varieties and Wasps for Their Use in Biological Control. The selection of varieties that are highly attractive to parasitoids or selection of parasitoids that are particularly responsive to specific odors could be used to enhance biological control programs, as suggested by Loughrin et al. (1995) and Bottrell and Barbosa (1998). The exploitation of parasitoid associative learning in pest management has also been suggested (Wardle and Borden, 1985; Lewis and Martin, 1990; Papaj and Vet, 1990; Vet and Groenewold, 1990).

Some of the Mexican maize varieties tested here appear to be more attractive than others. Field studies will demonstrate if these differences in attractiveness are reflected in parasitism rates. The high genetic variability observed in odor emissions among maize lines (Gouinguené et al., 2001) make it an ideal plant to optimize its attractiveness.

In summary, we confirmed that there are qualitative and quantitative differences in herbivore-induced odor blends among maize varieties. Both quality and quantity of the volatile blends were important for the attraction of inexperienced *C. marginiventris*. Based on the results, we speculate that green leaf volatiles, which are the most common volatiles produced by herbivore-damaged plants, could be important for the attraction of naïve generalist parasitoids such as *C. marginiventris*. More specific compounds, such as terpenoids, could be learned associatively during host encounters. However, further studies will have to establish precisely which compounds are responsible for the observed differences in attractiveness and if such differences can be used to enhance biological control.

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ODOR COMPOSITION OF PREFERRED (BUFFALO AND OX) AND NONPREFERRED (WATERBUCK) HOSTS OF SOME SAVANNA TSETSE FLIES

NICHOLAS K. GIKONYO,[1,*] AHMED HASSANALI,[1] PETER G. N. NJAGI,[1] PETER M. GITU,[2] and JACOB O. MIDIWO[2]

[1]*International Centre of Insect Physiology and Ecology*
P.O. Box 30772, Nairobi, Kenya

[2]*Department of Chemistry, University of Nairobi*
P.O. Box 30197, Nairobi, Kenya

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Abstract—A previous study on the feeding responses of tsetse flies, *Glossina morsitans morsitans*, implicated the existence of allomonal barriers, both volatile and nonvolatile, on the nonpreferred host, waterbuck, *Kobus defassa*. In the present study, electroantennogram-active compounds in odors from waterbuck were compared with those of two preferred hosts of tsetse flies, buffalo, *Syncerus caffer*, and ox, *Bos indicus*. Odors from the three bovids were trapped on activated charcoal and/or reverse-phase (octadecyl bonded) silica and analyzed with a gas chromatography-linked electroantennographic detector (GC-EAD) and, where possible, identified by using gas chromatography-linked mass spectrometry (GC-MS) and chromatographic comparisons with authentic samples. The GC-EAD profiles (with *G. m. morsitans* antennae) of the odors of the two preferred hosts were comparable, comprising medium-chain, saturated or unsaturated aldehydes and phenols, with buffalo emitting a few more EAG-active aldehydes. Waterbuck odor gave a richer profile, consisting of fewer aldehydes but more phenolic components and a series of 2-ketones (C_8–C_{13}) and δ-octalactone. This bovid also emits moderate amounts of C_5–C_9 straight-chain fatty acids, some of which were detected in buffalo and ox only in trace amounts. However, these did not elicit significant GC-EAD responses. Waterbuck profiles from the antennae of *G. pallidipes* showed broad similarity to those from *G. m. morsitans*, although the composition of aldehydes and ketones was somewhat different, indicating species-specific difference in the detection of host odors. Certain waterbuck-specific EAG-active components, particularly the 2-ketones and lactone, constitute a candidate allomonal blend in waterbuck odor.

* To whom correspondence should be addressed. E-mail: ngikonyo@icipe.org

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Key Words—*Glossina morsitans morsitans*, *G. pallidipes*, tsetse, buffalo, ox, waterbuck, host preferences, repellents, attractants, allomonal barrier.

INTRODUCTION

Blood-meal analyses of different species of tsetse flies (*Glossina* spp.) have shown varying degrees of specialization on specific groups of vertebrate hosts (Weitz, 1963; Vale, 1974a; Turner, 1987; Snow et al., 1988: Moloo, 1993; Clausen et al., 1998). For example, in the Luangwa valley of Zambia, *Glossina morsitans morsitans* and *G. m. centralis* derive over 90% of their blood meals from warthog (*Pharcochoerus aethiopicus*), hippopotamus (*Hippopotamus amphibius*), bushpig (*Potamochoerus porcus*), cattle (*B. indicus*), bushbuck (*Tragelaphus scriptus*), elephant (*Loxodonta africana*), and buffalo (*S. caffer*); other mammals in the area such as black rhinoceros (*Diceros bicornis*), zebra (*Equus burchelli*), giraffe (*Giraffa camelopardalis*), waterbuck (*K. defassa*), hartebeest (*Alcelaphus buselaphus*), and impala (*Aepyceros melampus*) are rarely fed on (Clausen et al., 1998). Similarly, in Lambwe Valley, Kenya, where *G. pallidipes* is the dominant species, over 80% of feeding is on bushbuck, buffalo, and bushpig, but none occurs on common game such as oribi (*Ourebia ourebia*), impala, waterbuck, reedbuck (*Redunca redunca*), and hartebeest (Turner, 1987). Vale (1974b) observed that very few *G. m. morstans* land and engorge on impala compared to ox, bushpig, and warthog. Similarly, in enclosures with ox, buffalo, eland (*Taurotragus oryx*), oryx (*Oryx beisa*), and waterbuck, *G. pallidipes* preferred to alight and engorge on buffalo or ox, followed by eland and oryx; few flies were associated with waterbuck, and none were seen to engorge (Grootenhuis, 1986; Grootenhuis and Olubayo, 1993).

Tsetse flies locate potential hosts beyond their visual range upwind (60–120 m) through odor-mediated anemotaxis, with visual cues becoming important at close range (≈10 m) (Vale, 1974b, 1977; Gibson and Torr, 1999). Near or on a host, thermal, tactile, and short-range or contact chemical stimuli induce a sequence of behaviors, including landing, probing, and feeding, which may contribute to host acceptability (Reinouts van Haga and Mitchell, 1975; Packer and Warnes, 1991; Van der Goes van Naters et al., 1998; Gikonyo et al., 2000). Some of the long-range kairomone components used by savanna tsetse fly species to locate their hosts are known. They include breath constituents, such as carbon dioxide, acetone, and 1-octen-3-ol (Hall et al., 1984; Vale and Hall, 1985), and phenolic microbial breakdown products (particularly, 4-cresol and 3-*n*-propylphenol) of host skin secretions and urine (Hassanali et al., 1986; Owaga et al., 1988; Bursell et al., 1988; Vale et al., 1988; Warnes, 1990; Saini et al., 1993). Different combinations of these attractants have been successfully deployed in the development of bait technologies for the control of *G. pallidipes* and *G. m. morsitans* (Vale et al., 1988; Brightwell et al., 1991; Willemse, 1991).

Madubunyi et al. (1996) found no significant differences in the phenolic composition and attractiveness to tsetse flies of urine samples derived from waterbuck, buffalo, and cattle. Therefore, Gikonyo et al. (2000) hypothesized that in nonpreferred vertebrates, key skin-derived kairomone components may be absent (or present in suboptimal amounts) or allomones may be present. Their observations of the responses of caged teneral *G. m. morsitans* on waterbuck and ox and on feeding membranes with and without smears of waterbuck sebum suggested the presence of both volatile and nonvolatile allomones on waterbuck skin. Our objective was to determine if the odor composition, particularly the GC-EAD profile, of waterbuck with respect to *G. m. morsitans* differs significantly from those of buffalo and ox. In addition, we compared the GC-EAD profiles of *G. m. morsitans* and *G. pallidipes* with respect to waterbuck odor.

METHODS AND MATERIALS

Host Animals. Waterbucks and buffaloes were born and reared at the Wildlife Disease Research Center, Kenya Agricultural Research Institute, Kabete, in an area free of tsetse flies. They were maintained on hay and concentrates (livestock feed pellets) and grazed freely within pens. Oxen were kept within pens at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, and fed on hay and concentrates. The animals were maintained free of acaricides for more than one year.

Insects. G. m. morsitans and *G. pallidipes* were reared at the ICIPE insectary under a 12L:12D photoperiod, $25 \pm 2^{\circ}$C, $70 \pm 5\%$ relative humidity, and fed off rabbits' ears. *G. pallidipes* was also caught from the wild at Nguruman, southwest Kenya and maintained as above.

Odor Trapping. This was carried out conveniently with adsorbent sachets placed on the animals. Activated charcoal (350 mg, 80/100 mesh, Chrompack) and octadecyl bonded silica (450 mg, particle size 40 μm, J. T. Baker, Phillipsburg, New Jersey, USA), Soxhlet-cleaned with dichloromethane for 24 hr, and then dried at 40°C, were enclosed in sachets (4×4 cm) made of either filter paper (Whatman No. 1) or stainless steel mesh-wire sheet (250 mesh) folded from larger pieces (5×10 cm) and stapled securely together. Twenty such sachets were placed along mesh belts (8×41 or 126 cm) made from strips of the stainless steel mesh-wire sheet folded lengthwise in the middle (Figure 1). A strip of clean aluminum foil formed the outer part of the belt. Adsorbent sachets were arranged at intervals above the wire mesh along the inside of the belt, which was hemmed and stapled tightly on one side. The sachets were, thus, exposed to air on the inner side, and sealed by the aluminum foil on the other. The two ends of the belt (about 5 cm) were folded and stapled to provide a loop through which sisal twine (also Soxhlet cleaned with dichloromethane) would pass for tying the belt around an animal (Figure 2). The belts containing the sachets were Soxhlet cleaned with

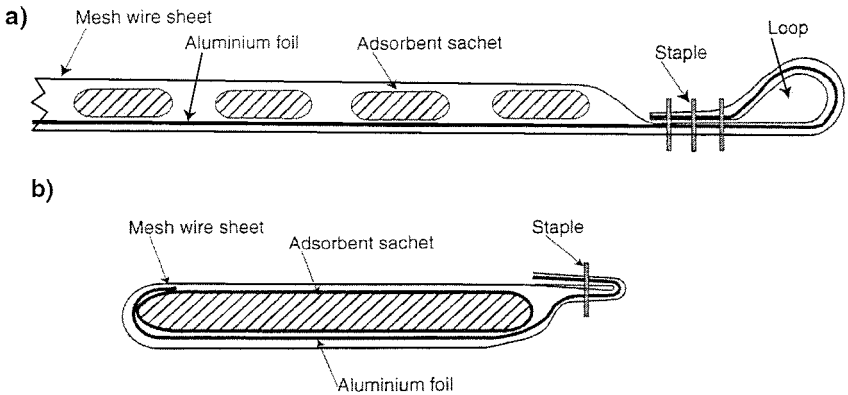


FIG. 1. Longitudinal (a) and cross-sectional (b) representation of adsorbent belt.

dichloromethane for 24 hr and then dried overnight at 40°C in a clean oven. The belts were transported wrapped lengthwise in aluminum foil and immediately tied around the neck and belly near the front and hind legs of animals restrained in a metallic crush (Figure 2). The belted animals were then released into pens to feed freely for the rest of the day. Because buffaloes tended to remove the belts tied round their bellies, only neck belts were used on them. All trapping was done from ca. 10:00–17:00 hr on sunny days when the average temperature was 26 ± 2°C and the relative humidity 40 ± 5%. Control belts were exposed to the air ambient around the crush for the same time taken to tie a single belt around an animal. After

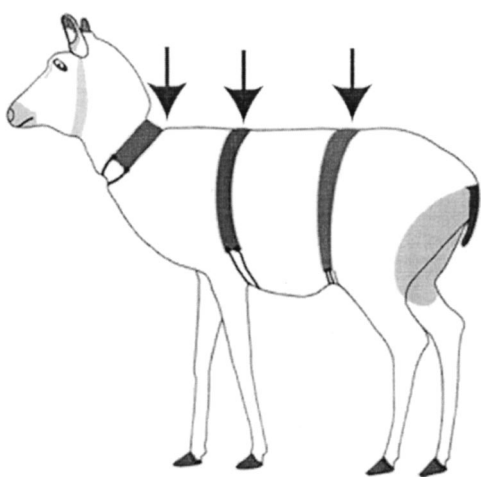


FIG. 2. Locations (arrows) of adsorbent belts on waterbuck.

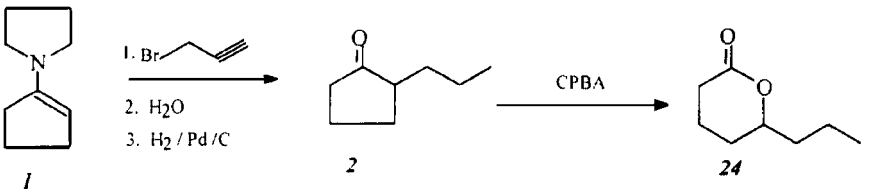
trapping, the animals were herded into the crush, and the belts removed, wrapped in clean aluminum foil, placed in a cool box, transported to the laboratory, and stored at −20°C.

Adsorbent from two sachets was pooled and eluted with 1 ml redistilled dichloromethane (over 99.9%; GC). The eluent was collected in a clean 4-ml vial, concentrated in ice under a gentle stream of clean nitrogen to 20 μl, and stored at −20°C.

Chemical Analyses. Gas chromatographic (GC) analyses were carried out on a Hewlett Packard (HP) model 5890 Series II gas chromatograph equipped with a splitless capillary injector system, a flame ionization detector (FID), and an HP 3396 Series II integrator. The GC oven was fitted with an HP fused silica capillary column (50 m × 0.2 mm × 0.33 μm) and temperature programmed at 40°C for 15 min, 8°C/min to 280°C, and held for 15 min. GC-MS analyses were carried out on a VG Masslab 12-250 mass spectrometer (EI, 70 eV) coupled to an HP 5790 Series A gas chromatograph, which was operated under the same conditions as the GC analyses. The synthetic standard chemicals were purchased from Aldrich Chemicals.

Electroantennographic Analyses (GC-EAD). Antennae were prepared from 3-day old adult female laboratory-reared *G. m. morsitans* and from laboratory-reared and field-trapped *G. pallidipes*. All flies had been fed two days earlier. A fly was held with fine forceps under a dissecting microscope and one of the antennae was gently pulled off with another forceps. The antenna was then cut with a scalpel at the base of the third broad segment into which a glass micropipet containing tsetse saline (Gee, 1976) was inserted. The micropipet with the antennal preparation was sheathed over a silver wire (0.5 mm diam) recording electrode on one arm of a micromanipulator (Syntech). The indifferent electrode was a similar glass micropipet, sheathed over a similar silver wire held on the other arm of the micromanipulator, and grounded through a wire. To complete the circuitry, the tip of the micropipet on the indifferent electrode was gently but firmly positioned on the intact end of the antennal preparation. The recording electrode was connected via a probe to a Universal AC/DC UN 05 amplifier (Syntech) and to a computer (PC) with an EAD card. The micromanipulator was positioned such that the antennal preparation was directly in the path of the effluent from the opening of the stimulus delivery tube connected to the GC.

GC-EAD was performed on an HP model 5890 Series II gas chromatograph equipped with an FID and an HP 3396 Series II integrator, and a splitter. The same conditions were employed as in the GC analyses. A makeup gas with a flow rate of 40 ml/min was added just before the split point to accelerate the effluent through 50-cm deactivated fused silica columns. The effluents for antennal detection were driven through a transfer line maintained at 150°C (Syntech THC-3) into a moistened air stream (25°C, 90% relative humidity, 4.0 ml/sec) and delivered over the antennal preparation via a stainless steel tube (5 mm ID). FID and EAD



SCHEME 1.

signals were monitored synchronously using a program on a GC-EAD interface card (Syntech) installed in a PC. Recordings were obtained from at least three antennae for each stimulus. EAG-active compounds were initially identified from their mass spectra and then confirmed by coinjections of authentic compounds and trapped odors into GC, GC-EAD runs on *G. m. morsitans* or *G. pallidipes*, and GC-MS comparisons.

Synthesis. δ-Octalactone (**24**), identified from waterbuck volatiles was prepared according to Scheme 1, adopted from Fieser and Fieser (1967) and Furniss et al. (1991). 1-Pyrrolidino-1-cyclopentene (**1**) was allowed to react with propargylbromide in acetonitrile, and the resulting 2-propylcyclopentanone (**2**) was converted to the lactone using *m*-chloroperbenzoic acid (CPBA). The structure of δ-octalactone (**24**) was confirmed by MS, ^{1}H and ^{13}C NMR spectra. The synthetic product (an enantiomeric blend) was EAG-active on both *G. m. morsitans* and *G. pallidipes* and coeluted in GC with the target peak in the waterbuck odor.

RESULTS

Odor Trapping and Composition. GC analysis of odor trapped from waterbuck body surface showed that activated charcoal and reverse-phase silica were equally effective, although the relative proportions of some peaks were different. Subsequent trapping employed reversed-phase silica. The GC profiles from the three body regions (Figure 2) were comparable. Odor profiles of volatiles from three different waterbucks were also similar and did not change significantly during a four-year period. Apart from some quantitative differences, odor profiles of buffalo and ox during the study period were also comparable.

GC-EAD Analyses. Fourteen EAG-active constituents on *G. m. morsitans* were detected in the reversed-phase silica- and activated charcoal-trapped volatiles from the waterbuck (Figure 3, Table 1). The antennae of insectary-reared *G. pallidipes* did not respond to any waterbuck odor constituents, but field-trapped *G. pallidipes* yielded 13 EAG-active peaks (Table 1). Eleven and 10 EAG-active constituents were detected by antennae of *G. m. morsitans* in (reversed-phase silica-trapped) volatiles of buffalo and ox, respectively (Figure 3, Table 1).

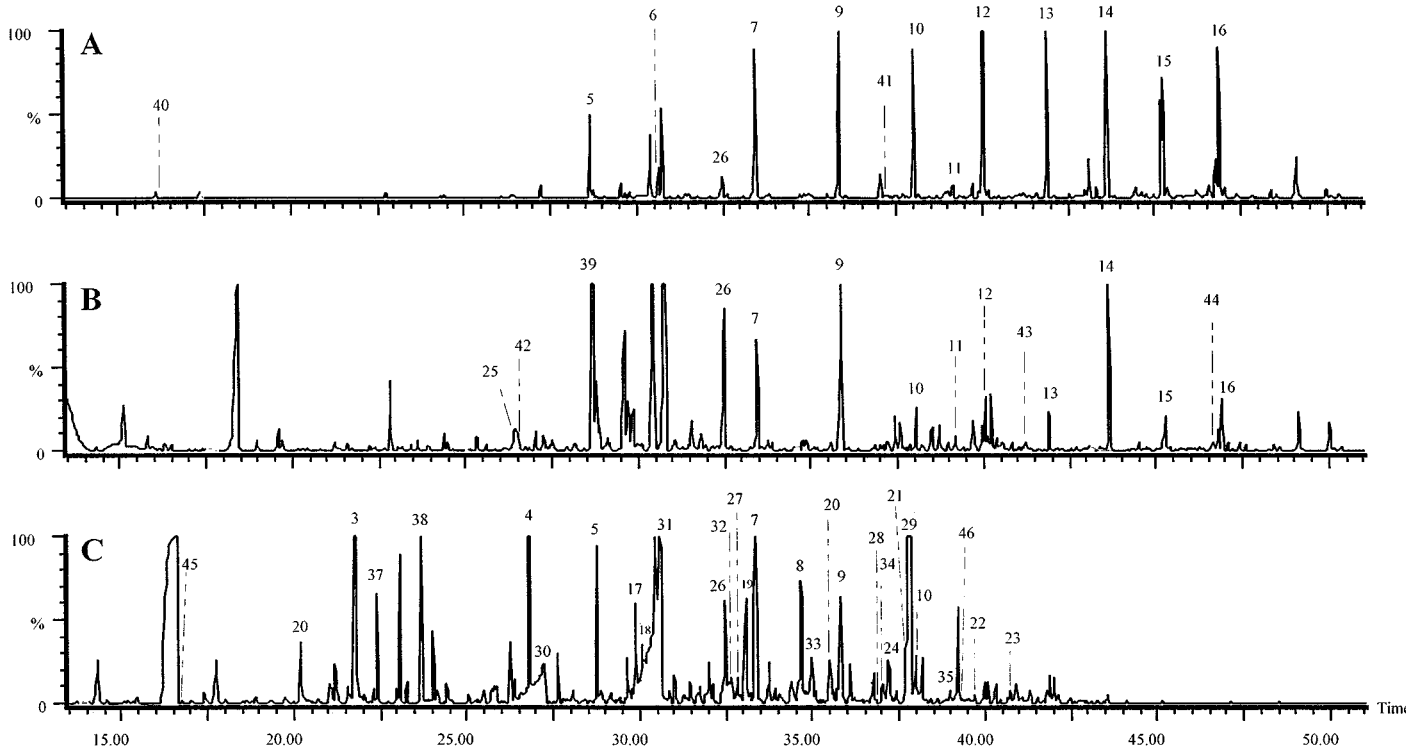


FIG. 3. Gas chromatograms of body odors from: buffalo (A), ox (B), and waterbuck (C). Numbered peaks refer to compounds in Table 1.

TABLE 1. COMPOUNDS DETECTED BY GC AND GC-EAD IN BODY ODORS OF BUFFALO, OX AND WATERBUCK[a]

Peak number	Compound	Presence in body odors		
		Buffalo	Ox	Waterbuck
3	Hexanal	−	−	+
4	Heptanal	+	−	+
5	(*E*)-2-Heptenal	+ (*)	−	+ (*†)
6	Octanal	+ (*)	−	−
7	Nonanal	+ (*)	+ (*)	+ (*†)
8	(*E*)-2-Nonenal	−	−	+ (†)
9	Decanal	+ (*)	+ (*)	+ (*†)
10	Undecanal	+ (*)	+ (*)	+ (*)
11	(*E*)-2-Undecenal	+ (*)	+ (*)	−
12	Dodecanal	+ (*)	+ (*)	−
13	Tridecanal	+	+	−
14	Tetradecanal	+	+	−
15	Pentadecanal	+	+	−
16	Hexadecanal	+	+	−
17	6-Methyl-5-hepten-2-one	−	−	+
18	2-Octanone	−	−	+ (*†)
19	2-Nonanone	−	−	+ (*†)
20	2-Decanone	−	−	+ (*)
21	2-Undecanone	−	−	+ (*)
22	2-Dodecanone	−	−	+ (*)
23	(*E*)-6,10-Dimethyl-5,9-Undecadien-2-one	−	−	+ (*†)
24	δ-Octalactone	−	−	+ (*†)
25	Dimethyl sulfone	−	+	−
26	4-Methylphenol	+ (*)	+ (*)	+ (*†)
27	2-Methoxyphenol	+	+	+ (†)
28	3-*n*-Propylphenol	+ (*)	+ (*)	+ (*†)
29	3-Isopropyl-6-methylphenol	−	−	+ (*)
30	Pentanoic acid	−	−	+
31	Hexanoic acid	−	−	+
32	Heptanoic acid	−	−	+
33	Octanoic acid	−	−	+
34	Nonanoic acid	−	−	+
35	Decanoic acid	−	−	+
36	Toluene	−	−	+
37	1-Octene	−	−	+
38	(*E*)-4-Octene	−	−	+
39	3-Ethyl-2-methyl-1-heptene	−	+	−
40	Unidentified	+ (*)	−	−
41	Unidentified	+ (*)	−	−
42	Unidentified‘	−	+ (*)	−
43	Unidentified	−	+ (*)	−
44	Unidentified	−	+ (*)	−
45	Unidentified	−	−	+ (†)
46	Unidentified	−	−	+ (†)

[a] +, detected; −, undetected. *EAG-active on *G. m. morsitans*; †EAG-active on *G. pallidipes* (waterbuck only).

Characterization of Waterbuck Odor. The 14 EAG *(G. m. morsitans)* active components of waterbuck odor (Figure 3A, Table 1) were identified by GC-MS, comparison of their mass spectral data with library data (NIST Registry of Mass Spectral Data), and GC coinjections as: **5**, (*E*)-2-heptenal, *m/z* (%) at 112 (4), 105 (6), 97 (8), 83 (66), 70 (36), 69 (35), 68 (33), 57 (51), 56 (46), 55 (68), 43 (35), 41 (100), 39 (52); **7**, nonanal; **9**, decanal; **10**, undecanal; **18**, 2-octanone; **19**, 2-nonanone; **20**, 2-decanone; **21**, 2-undecanone; **22**, 2-dodecanone; **23**, (*E*)-6,10-dimethyl-5,9-undecadien-2-one, 194 (0.4), 176 (1), 161 (1), 151 (10), 136 (10), 125 (6), 107 (12), 93 (7), 69 (46), 53 (6), 43 (100), 41 (35), 39 (7); **24**, δ-octalactone, 142 (1), 124 (5), 114 (12), 100 (8), 99 (100), 96 (9), 85 (6), 71 (73), 70 (55), 56 (40), 55 (68), 43 (57), 42 (75), 39 (26); **26**, 4-methylphenol, 108 (81), 107 (100), 90 (9), 79 (22), 77 (27), 63 (5), 53 (11), 51 (12), 39 (13); **28**, 3-*n*-propylphenol 136 (38), 121 (16), 108 (37), 107 (100), 91 (6), 77 (23), 65 (5), 51 (7), 39 (11); and **29**, 3-isopropyl-6-methylphenol, 150 (41), 136 (12), 135 (100), 121 (4), 115 (10), 107 (13), 91 (21), 77 (11), 65 (5), 51 (6), 41 (5), 39 (6). Of the 13 components active on *G. pallidipes*, 11 were similarly identified as: **5**, (*E*)-2-heptenal; **7**, nonanal; **8**, (*E*)-2-nonenal, 122 (5), 111 (9), 98 (19), 97 (16), 84 (32), 83 (66), 70 (85), 69 (56), 67 (23), 57 (55), 55 (92), 43 (93), 41 (100), 39 (52); **9**, decanal; **18**, 2-octanone; **19**, 2-nonanone; **23**, (*E*)-6,10-dimethyl-5,9-undecadien-2-one; **24**, δ-octalactone; **26**, 4-methylphenol; **27**, 2-methoxyphenol, 124 (85), 109 (100), 95 (2), 81 (65), 65 (7), 63 (7), 53 (22) 39 (11); and **28**, 3-*n*-propylphenol. Two minor components, **45** and **46**, active on *G. pallidipes*, which overlapped with other compounds, could not be identified. Additional EAG-inactive waterbuck odor components identified by GC-MS and comparisons with library mass spectra are listed in Table 1.

Characterization of Buffalo and Ox Odors. Nine of the 11 constituents of buffalo odor active on *G. m. morsitans* were identified and authenticated as: **5**, (*E*)-2-heptenal; **6**, octanal; **7**, nonanal; **9**, decanal; **10**, undecanal; **11**, (*E*)-2-undecenal, 165 (0.01), 147 (0.02), 121 (2), 97 (6), 83 (12), 70 (23), 57 (34), 55 (45), 43 (52), 41 (100), 39 (46); **12**, dodecanal; **26**, 4-methylphenol; and **28**, 3-*n*-propylphenol. Seven of the 10 active compounds from ox odor, on *G. m. morsitans*, were identified and authenticated as: **7**, nonanal; **9**, decanal; **10**, undecanal; **11**, (*E*)-2-undecenal; **12**, dodecanal; **26**, 4-methylphenol; and **28**, 3-*n*-propylphenol. The structures of five other minor components, **40** and **41** from the buffalo odor, and **42**, **43**, and **44** from ox odor, active on *G. m. morsitans*, could not be characterized because of coelution with other compounds. EAG-inactive components, prominent in the buffalo and ox odor collections, were also identified by GC-MS and comparisons with library spectra (Table 1).

DISCUSSION

The results of Gikonyo et al. (2000) on the behavior of teneral *G. m. morsitans* in small experimental cages on waterbuck and ox, and on feeding membranes with

and without smears of waterbuck sebum suggested the presence of volatile and short-range or contact allomones on waterbuck. Our results provide a chemical basis for discrimination between volatiles of waterbuck and those of buffalo and ox by tsetse flies. Of the known tsetse fly attractants (Hassanali et al., 1986; Owaga et al., 1988; Bursell et al., 1988), 4-methylphenol and 3-*n*-propylphenol were common to all the three bovids, albeit in varying relative amounts. Previously, these compounds were also found in the fermented urine of these animals (Madubunyi et al., 1996). In addition, different numbers of EAG-active, medium-chain (C_7–C_{11}) aldehydes were present in the odor of these animals. A notable difference in the GC-EAD profiles is the presence of δ-octalactone, 2-methoxyphenol (guaiacol), 3-isopropyl-6-methylphenol, and a series of (C_8–C_{13}) methyl ketones in waterbuck odor that were either not detected or present in trace amounts in the two preferred hosts. Guaiacol is moderately repellent to tsetse flies (Vale et al., 1988; Torr et al., 1996). Although we found only trace amounts (GC-EAD) of guaiacol in buffalo and ox volatiles, as did Bursell et al. (1988) and Torr et al. (1996), its amount was four times higher than 3-*n*-propylphenol in the waterbuck odor. A number of medium-chain 2-ketones are repellent or toxic to different insects, e.g., the maize weevil *Sitophilus zeamais* (Lwande et al., 1992), ants and termites (McDowell et al., 1988), and beet armyworm *Spodoptera exigua* (Lin et al., 1987). In field studies, several C_6 and C_7 methyl ketones reduced tsetse fly catches on electric nets, although some of the lower homologs (C_3–C_4) were attractive (Vale, 1980). The ketones in waterbuck odor together with guaiacol and the other two EAG-active constituents, acting additively or synergistically, could potentially constitute a long-range allomonal barrier. We are currently evaluating blends of these compounds for repellency.

Another distinctive feature in the odor chemistry of waterbuck compared to that of buffalo and ox is the presence of moderate amounts of C_5–C_9 straight-chain fatty acids. These, together with isopropyl-6-methylphenol, impart the cloying, musky smell of waterbucks (Estes, 1991; Mihok et al., 1991), which may also deter predators such as lion, leopard, and hunting dogs (Haltenorth and Diller, 1996). In our GC-EAD runs, none of the fatty acids elicited a discernible EAG response. Paradoxically, some of these acids reduced tsetse fly trap catches in the field (Vale, 1980), as did pentanoic and hexanoic acids (Torr et al., 1996). When added to targets baited with either a blend of known tsetse fly attractants or natural ox odor, pentanoic acid had no effect on the number of flies attracted, but did have a close-range effect on reduced trap entry. It also caused a small but significant reduction in feeding efficiency on the host (Torr et al., 1996). How then do we reconcile the effect of these compounds on tsetse behavior in these studies and lack of responses in our GC-EAD runs? Several possibilities exist. In the relative amounts present on the animal, the acids may not be behaviorally relevant to the fly, and the field results may represent an artifact resulting from relatively high doses used. On the other hand, a different set of receptors in the fly, perhaps located

in the tarsi, may be involved. Alternatively, the C_5–C_9 acids may act additively or in concert at the antennal receptors that results in an escape behavior closer to the animal where higher relative amounts of the longer chain homologs may be present. We are currently examining the validity of these alternatives.

The occurrence of a different combination of EAG-active aldehydes in waterbuck volatiles, compared to similar blends in the two preferred hosts, suggests a possible role for these compounds. Vale (1980) screened individual C_1–C_7 aldehydes in the field and found none to be reproducibly attractive. However, these compounds have not been tested in host-specific blends. Indeed, natural ox odor is 1.5–2 times more attractive than combinations of presently recognized attractants (carbon dioxide, phenols, and C_3–C_4 ketones) delivered at rates comparable to those emitted by ox (Torr et al., 1996), suggesting yet unidentified attractive constituents.

The GC-EAD profiles of waterbuck odor with *G. m. morsitans* and *G. pallidipes* antenna show that these related savanna species do not respond electrophysiologically to the same set of odor components. Although both species responded to (*E*)-2-heptenal, nonanal, and decanal, only *G. m. morsitans* responded to undecanal, and only *G. pallidipes* to (*E*)-2-nonenal. Moreover, *G. m. morsitans* responded to six 2-ketone odor components, only three of which elicited responses from *G. pallidipes*. Likewise, *G. pallidipes* was insensitive to 3-isopropyl-6-methylphenol, which elicited a distinct response from *G. m. morsitans.* These results indicate the olfactory uniqueness of each species and may partly account for the observed gradation of preferences for particular hosts. For instance, although waterbuck is generally avoided by *Glossina* species, it is comparatively more often fed on by *G. pallidipes* than *G. m. morsitans*. Interestingly, the former detected fewer 2-ketones in the present study.

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TASTE SENSITIVITY OF DETRITIVOROUS MOSQUITO LARVAE TO DECOMPOSED LEAF LITTER

JEAN-PHILIPPE DAVID,[1] ANDRÉ FERRAN,[2] JACQUES GAMBIER,[2] and JEAN-CLAUDE MEYRAN[1,*]

[1]*EA 3112 "Ecosystèmes et Changements Environnementaux"*
Centre de Biologie Alpine, Université J. Fourier
BP 53, F-38041 Grenoble Cedex 9, France

[2]*INRA, UMR ROSE*
37 Bd. Du Cap
BP 2078, F-06606 Antibes Cedex, France

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Abstract—Dietary leaf litter chemistry is known to play an important ecotoxicological role in the plant–mosquito interaction in subalpine flooded areas surrounded by vegetation because of differential larvicidal effects of insoluble polyphenols formed during the leaf decaying process. This dietary interaction was investigated through comparative evaluation of the role of toxic/nontoxic leaf litter in both larval foraging and feeding behavior, by using different samples of decomposed alder leaf litter and larval *Aedes aegypti* as experimental references. Track analysis showed significant differences in larval foraging behavior in the absence or presence of leaf litter. Comparative alimentary preference investigations and further track analysis suggested that larvae are unable to detect leaf litter toxicity. These characteristics of the larval behavioral feeding pattern suggested that: (1) decomposed leaf litter may be involved as an important attractive food source in the habitat selection and evolutionary history of culicids, and (2) preingestive behavioral mechanisms appear to be minimally involved in the differential larval dietary adaptation to toxic leaf litter. These results may have interesting consequences for culicid biological control.

Key Words—Feeding behavior, track analysis, dietary preferences, alder leaf litter, larvicidal activity, resistance, *Aedes aegypti*.

* To whom correspondence should be addressed. E-mail: jcmeyran@ujf-grenoble.fr

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INTRODUCTION

The ability of phytophagous insects to tolerate plant secondary compounds is an important determinant of both ecological and evolutionary patterns in many terrestrial taxa (reviewed in Bernays and Chapman, 1994). These xenobiotics may act at two different steps in plant–insect dietary interactions. Before ingestion, they may act as deterrents, thus contributing to behavioral resistance (reviewed in Glendinning and Slansky, 1995; Bernays et al., 2000). After ingestion, they may induce digestive detoxification processes (Brattsten, 1992), thus allowing biochemical resistance. Polyphenols are among those vegetable xenobiotics that can act through either inhibition of feeding (reviewed in Appel, 1993) or direct toxicity against the midgut (reviewed in Barbehenn and Martin, 1994).

In subalpine mosquito breeding sites, polyphenols of dietary leaf litter originating from riparian vegetation were demonstrated to be involved in habitat choice (Rey et al., 2000) and evolutionary history (Rey et al., 2001a) of the detritivorous larval culicid fauna. Habitat selection among taxa and/or populations appeared to be the result of toxicity of these polyphenols versus the differential tolerance of the consumers to those dietary xenobiotics (David et al., 2000a; Meyran et al., 2002). The toxicity of leaf litter was effective after a complex 10-month decaying process in water, and correlated to the phenolic activity of an extractable cell wall fraction including ligninlike compounds (David et al., 2000b, 2001a). Ingested toxic leaf litter exerted deleterious effects on the midgut of larval mosquitoes (David et al., 2000a) and other larval Diptera (Rey et al., 1999a). The epithelial degenerative pattern was comparable to that generally described in the insect midgut in response to toxic dietary xenobiotics (e.g., vegetable polyphenols: reviewed in Steinly and Berenbaum, 1985; bacterio-insecticides: reviewed in Rey et al., 1998). Such acute larvicidal effects of dietary decomposed leaf litter may be used for a new insecticide strategy against anthropophilic, vector-competent, invasive culicid taxa (Rey et al., 2001b).

Comparative investigations among taxa and populations originating from vegetable environments with different polyphenol richness have revealed the involvement of biochemical mechanisms in the differential tolerance of culicid fauna to decomposed leaf litter. The ability of larvae to metabolize those dietary xenobiotics was correlated to levels of cytochrome P-450 monooxygenases (P-450) and esterases (David et al., 2000c, 2002).

Because leaf litter ingestion remains the starting point of this particular plant–mosquito chemical interaction, larval *Aedes* feeding behavior toward leaf litter was investigated in the current study. Our aim was to comparatively evaluate the role of decomposed toxic/nontoxic leaf litter in larval feeding behavior. This experimental approach will allow us to clarify whether behavioral factors may be involved in larval tolerance to toxic leaf litter.

METHODS AND MATERIALS

Insect and Plant Materials. The *Aedes*–alder (*Alnus glutinosa* (L.) Gaertn.) interaction was chosen as a reference for our investigations because of the predominance of this culicid genus in subalpine mosquito-breeding sites (Rey et al., 2000) surrounded by a canopy dominated by alder (Pautou and Girel, 2001). For convenience and to avoid variability inherent in field *Aedes* populations, experimentation was performed on a laboratory strain of *A. aegypti* L. raised under standard conditions (Rey et al., 1999b). Despite its tropical origin, *A. aegypti* is known to have a feeding mode and a dietary tolerance to alder leaf litter comparable to those of numerous subalpine *Aedes* taxa (David et al., 2000a; Chaton et al., 2001). Fourth-instars of equal size were used either after starvation for 20 hr in order to increase ingestion rate during feeding trials (Rashed and Mulla, 1989), or without starving.

Samples of alder leaf litter, taken from the same woodland submitted to periodic flooding in the Rhône-Alpes region (France) and known to have different toxicity levels (David et al., 2000b, 2001a), were assayed. Crude 10-month decomposed leaf litter, known to be toxic ($LD_{50} = 0.1$ g/liter) (David et al., 2000b) was used as the toxic sample. Two types of nontoxic samples were used: (1) crude one-month decomposed leaf litter, in which toxic polyphenols had not yet formed, and (2) autoclaved crude 10-month decomposed leaf litter, in which toxic polyphenols had been destroyed by heat (David et al., 2001a). In the preference bioassays, toxic and nontoxic samples were used with a fine texture (50–100 μm) obtained according to the technique of David et al. (2000b), except for one trial where toxic samples were used either with a coarse texture (>0.5 mm) or with a fine texture. In the track analysis experiments, fine leaf-litter suspensions, obtained by filtration of 500 mg crude leaf litter in 200 ml tap water, were used in order to increase video tracking quality.

Video Observation of Larval Feeding Behavior. To analyze their feeding behavior in the presence of leaf litter, 20 larvae were individually videotaped while feeding. Each larva was placed in a round glass dish (4 cm diameter) containing 2 ml of filtered tap water and a freshly cut 2-mm^2 piece of 10-month decomposed alder leaf litter. Each larva was allowed to acclimate for 5 min and then filmed for 5–30 min using a video camera (Canon EX1, 100-mm objective, macro mode). Images were recorded on a JVC SX120 videocassette and viewed on a Sony color monitor. After tape analysis, representative pictures of larval feeding behavior were selected using a video acquisition computer card and converted into black and white images.

Track Analysis. Because feeding by mosquito larvae requires ingestion processes and whole-body movements (reviewed in Merritt et al., 1992a,b), a track analysis of larval foraging behavior was performed on individual larvae placed into round glass dishes (18.6 cm diameter) containing 100 ml of filtered tap water

or filtrate of leaf-litter (i.e., toxic or nontoxic leaf litter sample). To avoid larval lighting disturbance (Olsson and Klowden, 1998), a 50-cm-high cylindrical sheet of white paper was put around the dish during experiments. Each larva was allowed to acclimate for 5 min in tap water, and then filmed for 1–5 min, depending on the number of recorded paths (1–5 paths/larva). The same larva was then transferred to leaf litter filtrate (toxic or nontoxic alternately) and filmed as previously described. All experiments were carried out under insectarium conditions and with constant white light, using a RCA black and white camera fitted out with a 35–200 objective mounted with a C-type adaptation ring. The paths followed by each larva were then analyzed with a SECAM acquisition video card and computed with Cible4 tracking and Harmony analysis software. Paths were automatically smoothed (average of five successive points) and all coordinates (25 frames/sec) were used.

Larval track was characterized by the linear speed (millimeters per second, excluding stop duration), the number of stops (per second), and the angular index (bearing variations along paths in degrees per second). Results were expressed as means (± standard deviation). Statistical analyses were done with Minitab sofware. Comparisons between absence/presence of leaf litter were obtained by paired data *t* test ($N > 75$, $df = N - 1$) because larvae were successively placed in two different situations (e.g., tap water and leaf litter filtrate). Comparisons between toxic/nontoxic leaf litter were obtained by a two-sample *t* test ($N_1 + N_2 > 75$, $df = (N_1 + N_2) - 2$) because different larvae were used for comparisons.

Dietary Preference Bioassays. Two hours before each bioassay, two homogeneous sets of 50 larvae were differently colored *in vivo* by using two alimentary dyes (E-122 for red, E-102 and E-131 for green) diluted in tap water. A clear Plexiglas container (35 × 24 × 13 cm), divided into two equal compartments by a sliding polystyrene divider (Figure 1) and containing 6500 ml of tap water, was allowed to stand for 30 min under insectarium conditions to equilibrate at 25°C. In order to prevent larvae from being attracted to dark substrates (see references in Clements, 1999) and from behavioral disturbance due to lighting effects (Olsson

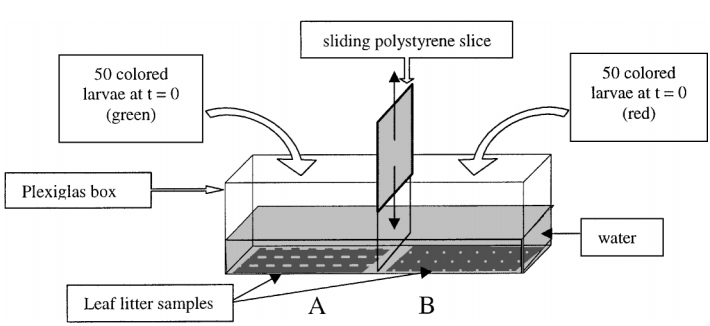


FIG. 1. Test arena for dietary preference bioassays.

and Klowden, 1998), a black sheet of paper was put under the Plexiglas box and a white one above the box. The effects of leaf-litter quality were compared by covering the floor of half the container with a toxic sample and the other half with a nontoxic sample (100 mg/assay) 10 min before introducing each set of 50 colored larvae into each compartment (Figure 1).

After allowing the larvae to disperse and equilibrate for 5 min in each compartment, the central polystyrene divider was pulled up, allowing the larvae to spread throughout the entire volume of water for 30 min. The central polystyrene divider was pulled down before identifying and counting the larval sets newly distributed into each compartment. Each assay was replicated three times.

Results were expressed as the percentage of total larvae present in the test arena (means ± SD). Statistical comparisons (one-way ANOVA) of the percentage of total larvae (without any distinction) present in each compartment after 30 min were made with Minitab software for each experiment.

RESULTS

Larval Leaf-Litter Feeding Behavior. Video observations of feeding larvae support the concept of arrestment in their food search strategy (Clements, 1999), as larvae randomly explore the aqueous environment before stopping at a leaf-litter piece which is recognized as a favorable food item. Moreover, the role of leaf litter as a direct food source is clearly demonstrated as larvae can be observed feeding directly on the leaf litter itself (Figure 2).

All observations show that larvae have a browsing feeding mode according to the terminology of Merritt et al. (1992a,b), and spend much time in shredding the

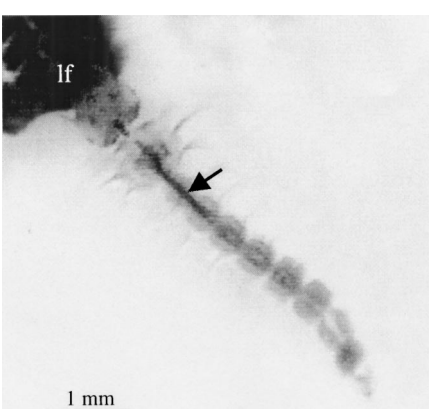


FIG. 2. *A. aegypti* larva browsing a piece of decomposed alder leaf litter (lf). Ingested leaf litter (arrow) is easily observable by transparency in the larval midgut.

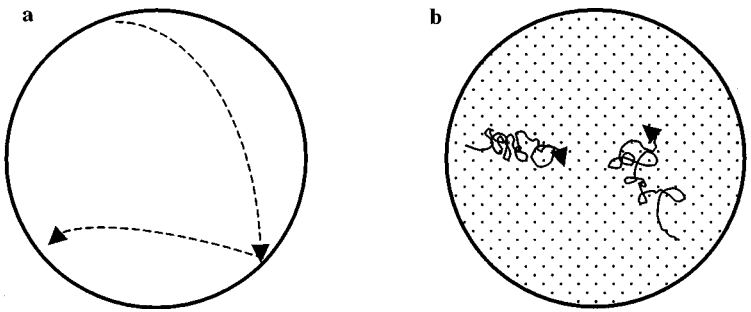


FIG. 3. Differences in larval *A. aegypti* track shapes in relation to food availability. Extensive search behavior, characterized by straight paths and high speeds are observed in filtered tap water (a), whereas intensive search behavior, expressed by curved paths associated with a slow gliding movement, can be observed in high food availability environment (b).

leaf litter piece. In the same way, because of the dark color of this food source, leaf-litter ingestion into the larval gut can be directly visualized through the larval body after 15 min of feeding (Figure 2). No postingestive regurgitation mechanisms, such as those reported for some larval Lepidoptera (Bernays et al., 1995), have ever been observed on leaf-litter feeding *Aedes* larvae.

Track Analysis. As shown in Figures 3 and 4, track analysis results reveal differential larval behavior in relation to environmental food availability. Without any food source (i.e., in filtered tap water), larvae show straight trajectories (Figure 3a) with a high average linear speed, low number of stops per second, and low rate of turning (Figure 4). During this extensive search behavior, larvae increase their linear speed and to explore a maximum area. This rapid progression is powered by the whole abdomen together with the respiratory siphon and the ventral terminal brush.

The same larvae then placed in an environment of high food availability (i.e., in rough leaf litter filtrates) show an instantaneous change in their behavioral characteristics. Trajectory shapes become very curved (Figure 3b) with a low linear average speed associated with high angular speed and high number of stops per second (Figure 4). During this intensive search-behavior, larvae optimize their ingestion rate as their mouthparts generate the currents propelling them. These currents (called α currents; Widahl, 1992) allow larvae to glide while gathering alimentary particles. The trajectory characteristics of this behavioral pattern are not different in the presence of toxic or nontoxic leaf litter ($P > 0.1$, $P > 0.06$ and $P > 0.1$ for average linear speed, average angular speed, and average number of stops respectively: Figure 4).

Dietary Preferences. Toxic leaf litter appears to be preferred to nontoxic litter by larvae, either when starving them first (data not shown) or not (Figure 5).

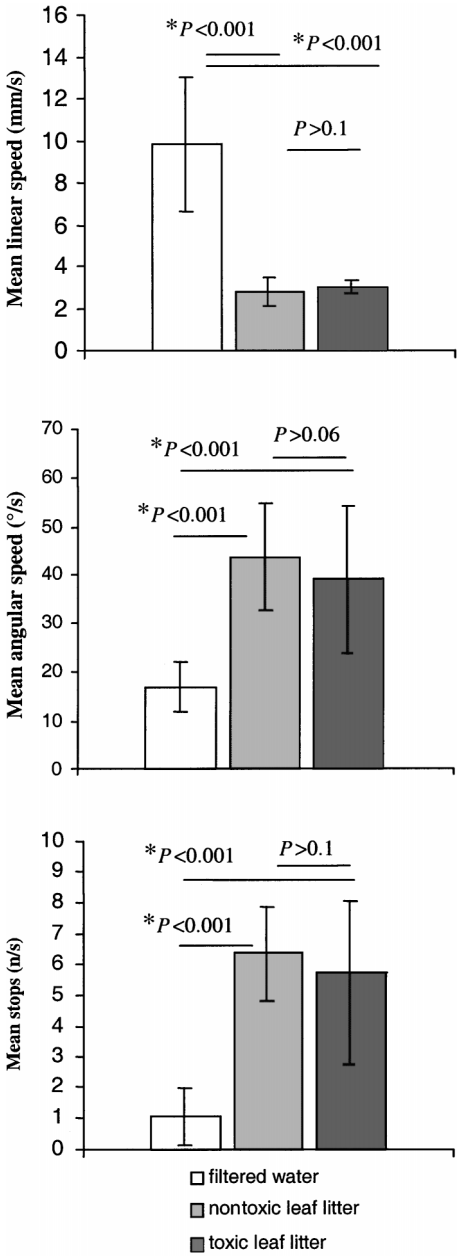
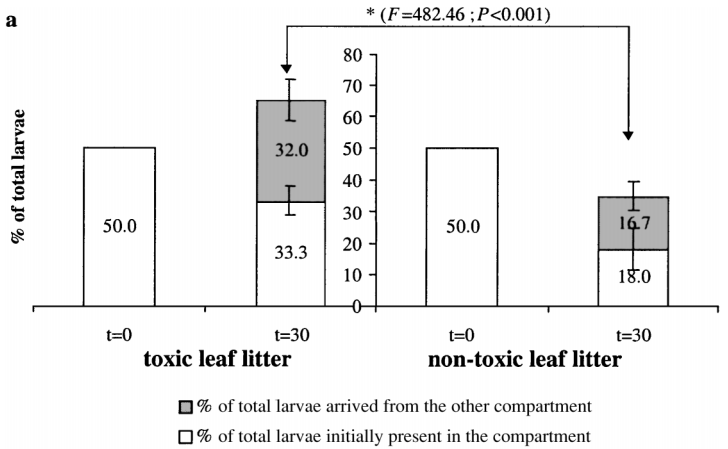
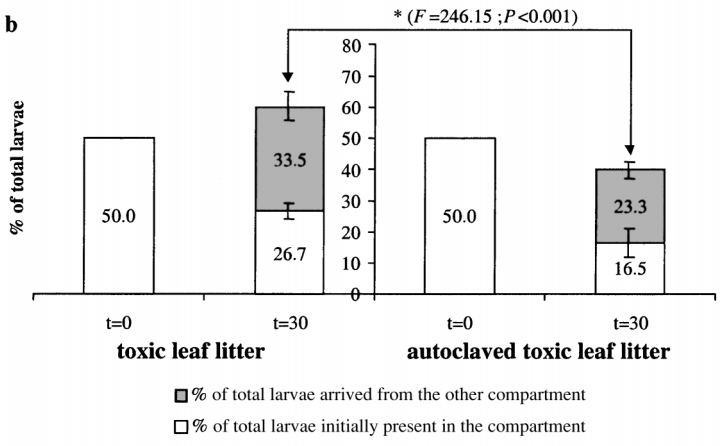
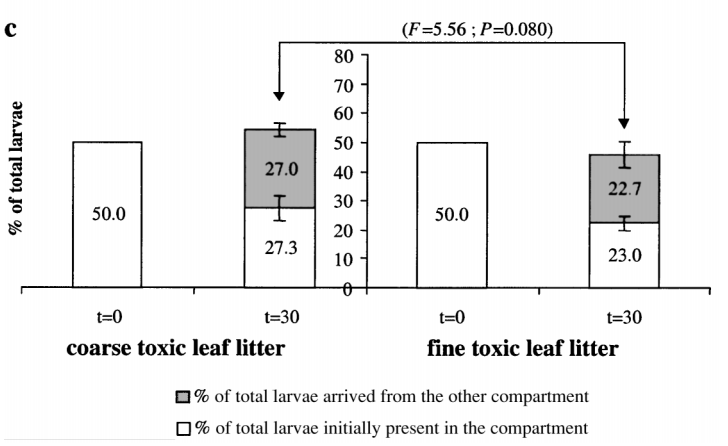


FIG. 4. Comparison of larval *A. aegypti* track characteristics in the absence of leaf litter (filtered water) and in filtrates of toxic and nontoxic leaf litter (mean ± SE). *P* values are given for all comparisons, $N > 75$, $df = -1$ or $(N + N_2) - 2$ for paired data *t* test and two-sample *t* test, respectively.


a
* (F =482.46 ; P<0.001)
% of total larvae
80
70
60
50
40
30
20
10
0
32.0
50.0
33.3
50.0
16.7
18.0
t=0
t=30
t=0
t=30
toxic leaf litter
non-toxic leaf litter
% of total larvae arrived from the other compartment
% of total larvae initially present in the compartment


b
* (F =246.15 ; P<0.001)
% of total larvae
80
70
60
50
40
30
20
10
0
33.5
50.0
26.7
50.0
23.3
16.5
t=0
t=30
t=0
t=30
toxic leaf litter
autoclaved toxic leaf litter
% of total larvae arrived from the other compartment
% of total larvae initially present in the compartment


c
(F =5.56 ; P=0.080)
% of total larvae
80
70
60
50
40
30
20
10
0
27.0
50.0
27.3
50.0
22.7
23.0
t=0
t=30
t=0
t=30
coarse toxic leaf litter
fine toxic leaf litter
% of total larvae arrived from the other compartment
% of total larvae initially present in the compartment

This was first demonstrated by the larval preference for crude 10-month decomposed leaf litter (i.e., including toxic polyphenols and with low mechanical resistance) versus crude one-month decomposed leaf litter (i.e., including no toxic polyphenols and with high mechanical resistance) (Figure 5a). In this case, more than 65% of total larvae were found in the toxic leaf litter compartment after 30 min of experiment. Moreover, among those 65% larvae found in the toxic leaf litter compartment, 32% were moving larvae originating from the nontoxic compartment, and 33.3% were arresting larvae originating from the toxic compartment.

Such a preference for toxic substrate was also found in the choice test between crude 10-month decomposed leaf litter (i.e., including toxic polyphenols and with low mechanical resistance) versus autoclaved 10-month decomposed leaf litter (i.e., where heat treatment destroyed toxic polyphenols and increased mechanical resistance) (Figure 5b). Thus, larval preference for toxic leaf litter appears to be related to its physical quality (i.e., related to the mechanical resistance to browsing) rather than to its chemical quality (i.e., related to the toxic polyphenol content).

Leaf-litter particle size does not seem to be a critical factor in larval dietary preferences, as coarse and fine pieces of toxic leaf litter appear to be equally attractive (Figure 5c), and both are browsed and ingested by larvae.

DISCUSSION

The current study suggests a role of leaf litter in structuring both feeding and foraging behavior of larval *Aedes* which appear unable to detect leaf-litter toxicity before ingestion. These behavioral characteristics of larval *Aedes* toward leaf litter may suggest a weak involvement of behavioral mechanisms in larval adaptation to the toxic polyphenols of dietary leaf litter. This may have important consequences in shaping the ecological and evolutionary patterns of *Aedes* populations in the subalpine mosquito breeding sites. It also reinforces the practicality of using decomposed alder leaf litter in a new larvicidal strategy.

Pattern of Leaf-Litter Feeding in Larval Aedes. Like numerous *Aedes* taxa, larval *A. aegypti* can have a browsing feeding mode focusing on a large spectrum of food sources (reviewed in Merrit et al., 1992a,b). Thus, it is not surprising that, as

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FIG. 5. Comparison of larval *A. aegypti* dietary leaf litter preferences, obtained by larval distribution between two types of leaf litters after 30 min of experiment. Results are expressed by percentage of total larvae in each compartment (mean $\pm$ SE), between toxic (i.e., crude ten-month decomposed) and nontoxic (i.e., one-month decomposed) leaf litters (a), between toxic and nontoxic (i.e., toxic autoclaved) leaf litters (b), and between toxic leaf litter with coarse and fine granulometry (c). P and F values are given for each comparison (one-way ANOVA, $N = 3$, $^*P < 0.001$).

suggested by our trajectory results, the presence of leaf litter in the aquatic medium instantaneously generates intensive foraging behavior by larvae. Such differences in foraging behavior according to the presence/absence of food in the medium is commonly observed among larval mosquito taxa where food generally acts as an arrestant (reviewed in Clements, 1999). Alder leaf litter also may be recognized by the larvae as a phagostimulant during browsing (Chaton et al., 2001). This attests to the generalized flexible feeding strategy of *Aedes* larvae whose plasticity is shaped mainly through environmental cues of food availability (Eisenberg et al., 2000).

In subalpine *Aedes* breeding sites, the prominence of leaf litter as a food source for mosquito larvae is easily understood because of the important annual leaf input from the riparian vegetation into the water (Pautou and Girel, 2001). Leaf litter appears to be directly used as a food source by *Aedes* larvae rather than its microbial content. This is clearly shown by both our current video observations and previous histopathological investigations within the midgut (David et al., 2000a), and is consistent with that known for other culicid larvae in different lentic habitats (Léonard and Juliano, 1995).

Despite the prominent role of this food source in larval *Aedes* feeding mode, there is no behavioral evidence for immediate sensory discrimination of leaf litter toxicity by larvae, whether or not they have been starved. Such an absence of relation between food selectivity and starvation contrasts to that observed for other phytophagous insects (Bernays and Chapman, 1994). Moreover, this lack of food selectivity contrasts to that observed for other culicine taxa where feeding behaviors include chemotaxis and chemokinesis mechanisms (reviewed in Clements, 1999). Larval preference for toxic leaf litter may be due to its physical quality in relation to the degree of leaf decomposition. Together with the induction of toxicity within the leaf litter (David et al., 2000b), the decaying process may reduce the mechanical resistance of leaf material to larval browsing (Cummins and Klug, 1979), thus enhancing its edibility (Gessner, 1991). The absence of any deterrent effect of the leaf-litter toxic chemicals against larvae may be explained in two different ways: (1) the insolubility of these polyphenols in the aquatic medium (David et al., 2000b, 2001) preventing their accessibility to the larval sensory apparatus, and (2) the absence of elaborate mechanisms for larval recognition of those molecules. Thus, toxic polyphenols may contribute little to structuring the larval pattern of feeding in the leaf litter–*Aedes* interaction. This contrasts to that observed in numerous phytophagous insect–plant interactions (e.g., Orthoptera: Bernays et al., 1995; lepidopteran caterpillars: reviewed in Bernays et al., 2000) where the presence of plant secondary compounds can affect foraging activities and influence diet choice.

Ecotoxicological and Applied Consequences of Leaf-Litter Feeding Behavior. As larvae appear able to discriminate only the physical quality of the leaf litter, the involvement of behavioral mechanisms in the larval adaptation to toxic leaf

litter may be minimal. Thus, dietary adaptation to leaf-litter polyphenols may have proceeded mainly through metabolic changes. This biochemical resistance appears to be mediated by P-450 and esterase activities (Rey et al., 1999b; David et al., 2000c, 2002), which allow the larvae to tolerate toxic leaf litter after ingestion. This stands in contrast with that observed, for example, in phytophagous insects where adaptation to toxic dietary plant secondary compounds is primarily mediated at a preingestive behavioral step (reviewed in Glendinning and Slansky, 1995; Bernays et al., 2000).

Thus, in subalpine mosquito-breeding sites, the selection indirectly exerted by the riparian vegetation on larval culicine fauna through dietary leaf-litter polyphenols may have generated only postingestive adaptative responses. Because of the predominance of a polyphenolic-rich leaf litter in a given breeding site, the larvae may have no possible diet choice. Thus, the acquisition of metabolic detoxification processes may have appeared as the main way for larval tolerance to those ingested leaf-litter toxic polyphenols. Such a metabolic adaptation has been considered as the key innovation (Berenbaum et al., 1996) that may have promoted ecological diversification (David et al., 2000c) and shaped evolutionary history (Rey et al., 2001a) of mosquito taxa throughout the subalpine breeding sites surrounded by vegetation with different polyphenol richness.

The larval predilection for decomposed alder leaf litter, together with the fact that this toxic food cannot be recognized as deterrent by larvae, encourages us to propose the use of leaf litter as a new useful mosquito control agent against some anthropophilic taxa (Rey et al., 2001b). Contrary to many conventional insecticides, toxic alder leaf litter remains stable in the aquatic environment (David et al., 2000b, 2001) and is actively searched for and ingested by larvae. Further investigations are currently being performed in order to optimize this new promising larvicidal strategy.

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THE PUTATIVE ROLE OF BOTRYDIAL AND RELATED METABOLITES IN THE INFECTION MECHANISM OF *Botrytis cinerea*

A. J. COLMENARES, J. ALEU, R. DURÁN-PATRÓN, I. G. COLLADO,* and R. HERNÁNDEZ-GALÁN

Departamento de Química Orgánica
Facultad de Ciencias, Universidad de Cádiz
República Saharaui s/n, Apdo. 40
11510 Puerto Real, Cádiz, Spain

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Abstract—Phytotoxic assays, performed both *in vitro* and *in vivo* on leaves of *Phaseolus vulgaris*, with metabolites excreted by the fungus *B. cinerea* are evaluated. Exogenous application of the phytotoxin botrydial has been found to produce severe chlorosis and cell collapse and facilitated fungal penetration and colonization of plant tissue. The results also show a light-dependent action mechanism for the phytotoxin and seem to indicate that botrydial is a non-host-specific toxin involved in fungal infection of *B. cinerea*.

Key Words—Botryane, botrydial, chlorosis, phytotoxin, *Botrytis*, virulence factor.

INTRODUCTION

A substantial number of toxins, produced as mixtures of either related or unrelated substances, have been extracted from cultures of many different bacterial and fungal pathogens. In fact, the ability to produce one or more toxins and to use them as chemical weapons may be the decisive factor in the virulence of plant pathogens, and perhaps even their pathogenicity (Rudolph, 1990). A limited number of toxins, the so-called host-selective toxins, affect only plants of one specific genotype susceptible to the pathogen. At low concentrations, these toxins reproduce the symptoms of natural infections. By far, however, the majority of phytotoxins are nonselective and do not reproduce the patterns of resistance and susceptibility of

* To whom correspondence should be addressed. E-mail: isidro.gonzalez@uca.es

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the host to the pathogen. These include all the bacterial phytotoxins described thus far and the majority of fungal toxins. Because these biologically active substances are broad-spectrum toxins that can affect many economically important crops, their total impact on agriculture has serious repercussions (Graniti, 1991).

Botrytis cinerea, the causative agent of the devastating gray mold disease in many economically important crops, produces characteristic metabolites with a botryane skeleton (Durán-Patrón et al., 1999). The role of these metabolites in the physiology of the fungus was hitherto unknown. Nevertheless, in previous work (Collado et al., 1996; Rebordinos et al., 1996; Durán-Patrón et al., 1997) we reported the phytotoxic effects of some of these compounds when they were assayed *in vitro* on leaf discs of *Nicotiana tabacum.*

Recently, the fungal metabolite botrydial was detected for the first time in plant tissues infected with isolates of *B. cinerea* (Deighton et al., 2001). The phytotoxin was produced in the soft rot regions of the infection. While it has not been ruled out that other toxins with a botryane skeleton may also be present in the tissues, the methodology of this particular study was applicable only to botrydial.

We have long felt that a greater understanding of the role of this toxin and its related compounds in the processes leading to disease onset and lesion expansion would be gained if the action mechanisms of the toxins involved were known. This paper describes experiments designed to determine the role of these compounds in the pathogenicity of the fungus *B. cinerea*. Phytotoxic assays with metabolites with the botryane skeleton (Figure 1) extracted from cultures of *B. cinerea* were studied. These assays were performed both *in vitro* and *in vivo* on leaves of *Phaseolus vulgaris*, as well as on different fruits. The effect of light on the action mechanism of botrydial was also studied.

METHODS AND MATERIALS

General. The strain *B. cinerea* Pers.: Fr. 2100 employed in this work was obtained from the Colección Española de Cultivos Tipos (CECT), Facultad de Biología, Universidad de Valencia, Spain. The procedures used for the isolation and purification of compounds **1–6** (Figure 1) have been described previously (Durán-Patrón et al., 1999). Pure metabolites were dissolved in 40% acetone (v/v) in 100 ml of water containing 10 μl of Tween 80. Solutions of different concentrations, ranging from 10 to 1000 ppm, were then prepared. Controls consisted of mixtures of aqueous acetone that had been used for dissolving the products.

Plant Material and Methods of Culture. Seeds of *Phaseolus vulgaris* genotypes N90598 (susceptible to *B. cinerea*) and N90563 Huron (resistant to *B. cinerea*) (Kelly et al., 1994) were obtained from J. D. Kelly, Michigan State University, East Lansing, Michigan, USA, and were grown in a standardized and quality controlled compost/sand mixture in individual pots in a greenhouse for two or three weeks at 25°C.

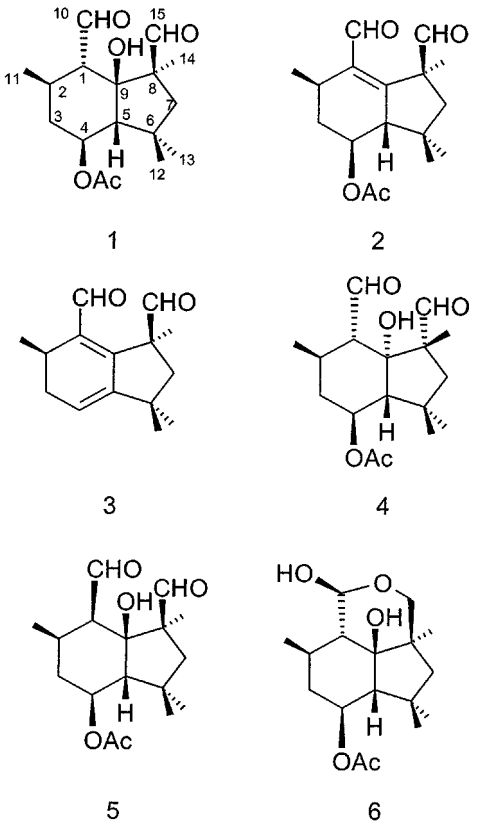


FIG. 1. Structures of compounds **1–6**.

Leaf Disc Assays. Leaves of *Phaseolus vulgaris* plants were sterilized with 10% bleach (v/v) for 3 min., washed four times with sterile water, dried between filter paper, cut into circles measuring 1 cm diam., and placed in Petri dishes (10 circles per dish) lined with Whatman paper wetted with sterile water. Each circle received a 10 μl droplet of pure compound solutions. The dishes were then incubated at 25–28°C in natural sunlight. Assays were carried out in duplicate.

The frequency of infection was ascertained by comparing the number of affected circles to the total number treated. The size of the damaged area was obtained by determining the affected surface area as compared with the total surface area, expressed in square millimeters.

Whole Plant Assays. Attached leaves were sterilized with an aqueous solution of 10% of ethanol (v/v), washed with sterile water, and dried between filter paper. In general, two *Phaseolus* plants were used per experiment. Five leaves per plant

were treated with a solution of the compound to be tested. Each leaf received five drops (10 μl) of control and five drops (10 μl) of the test solution. Two experiments were carried out for each compound and concentration. The plants were incubated at 25–28°C in natural sunlight for a minimum photoperiod of 12 hr. The diameter of the drops was identical in each case, and the total surface area of the drops was considered the treated area. The total treated area was considered to be 100%, and the affected area was calculated in relation to treated area.

Fruit Assays. Fruits (*Capsicum annuum*, *Licopersicom esculentum*, *Vitis vinifera*, *Phaseolus vulgaris*, *Fragaria vesca*, *Actinidia chinensis planchon*) were sterilized as described above and placed in Petri dishes lined with Whatman paper wetted with sterile water. Botrydial was dissolved to different concentrations (50–250 ppm) and was placed in 10 μl drops on the fruit surface. The treated fruit, five pieces per assay, was incubated at 25–28°C in natural sunlight. Two experiments were conducted for each type of fruit.

Inoculation Experiments. Sporulating fungal colonies of *B. cinerea* Pers.: Fr 2100 were first rinsed with an inoculating medium consisting of Gamborg's B5 obtained from Sigma Chemical CO (3.2 g/liter), sucrose (10 mM), and potassium phosphate buffer (0.01M, pH 6.4) and then filtered through cheesecloth to eliminate mycelial fragments. The conidial suspension was centrifuged, resuspended in an inoculating medium containing sucrose, and adjusted to approx. 10^5 conidia/ml. Five l-microliter droplets of conidial suspension were placed onto the leaf surfaces, and the leaves or plants were incubated in a chamber at 25°C in natural sunlight and with saturated air humidity.

The sunlight radiation was measured with the aid of a pyranometer (silicon cell pyranometer, SKS1110). An average total radiation of 514 W/m^2 and 360 W/m^2 was achieved, at plant level, for sunny and cloudy days, respectively.

RESULTS AND DISCUSSION

In an initial survey, we compared the activities of the phytotoxic metabolites produced by *B. cinerea in vitro* by incubating aliquots of solutions of the metabolites on leaf discs of *P. vulgaris* (genotype N90598) at concentrations of 1000, 500, 250, and 100 ppm. The more active metabolites were tested at concentrations under 100 ppm. The leaf circles were examined each day. The maximum phytotoxic effect was reached at different times for each metabolite, and remained constant thereafter. By the seventh day, the maximum effect for all metabolites had been reached (see Table 1; note that only those concentrations that gave a positive result are listed). Botryendial (**2**), botrydienal (**3**), and 8,9-epibotrydial (**4**) were highly phytotoxic; they produced chlorotic lesions at concentrations of 25, 25, and 50 ppm, respectively. Botrydial (**1**) had the highest level of activity; it caused a chlorotic effect even at a physiological concentration (1 ppm). Metabolites **5** and **6** were moderately active at a minimum concentration of 250 ppm.

TABLE 1. FREQUENCY AND SEVERITY OF LESIONS CAUSED BY METABOLITES OF *B. cinerea* ON *Phaseolus vulgaris* LEAF DISCS

Metabolite concentration (ppm)	Frequency of lesion (%)[a]	Severity of lesion (%)[b]
Botrydial (**1**)		
1000	100	100
500	100	100
250	100	90
100	100	76
50	100	40
10	80	15
1	30	<5
Botryendial (**2**)		
1000	100	100
500	100	68
250	100	30
100	100	15
50	100	13
25	100	9
Botrydienal (**3**)		
1000	100	100
500	100	73
250	100	45
100	100	34
50	100	20
25	100	12
8,9-Epibotrydial (**4**)		
1000	100	100
500	100	38
250	100	20
100	100	15
50	80	10
1-Epibotrydial (**5**)		
1000	100	20
500	100	16
250	90	11
Dihydrobotrydial (**6**)		
1000	100	24
500	100	20
250	100	14

[a] Arithmetic mean of the results from two independent experiments with 10 circles for each concentration in each experiment (standard deviation <3%).

[b] Calculated as the surface affected compared to the total surface expressed in mm^2.

TABLE 2. FREQUENCY AND SEVERITY OF LESIONS CAUSED BY METABOLITES OF *B. cinerea* ON *Phaseolus vulgaris* PLANTS

Metabolite concentration (ppm)	Frequency of lesion (%)[a]	Severity of lesion (%)[b]
Botryendial (**2**)		
1000	100	100
500	100	80
250	100	70
100	100	35
50	100	<5
25	50	<5
Botrydienal (**3**)		
1000	100	100
500	100	90
250	100	75
100	100	40
50	70	15
25	50	5
8,9-Epibotrydial (**4**)		
1000	100	100
500	100	95
250	100	90
100	100	20
50	90	5
1-Epibotrydial (**5**)		
1000	100	60
500	80	35
250	60	25
Dihydrobotrydial (**6**)		
1000	100	45
500	100	15
250	80	<5

[a] Arithmetic mean of the results from two independent experiments with 10 leaves for each concentration in each experiment (standard deviation <3%).
[b] Calculated as the surface affected compared to the total surface expressed in mm^2.

Compounds **1**–**6** were tested in whole-plant assays with *Phaseolus vulgaris*, genotype N90598. Although the correlation between *in vitro* and *in vivo* experiments was generally good, the phytotoxic effect was more rapid *in vivo*, being noticeable after only a few hours (Tables 2 and 3). It is worth noting that all compounds assayed caused severe chlorosis and cell collapse. Botrydial (**1**) was active from 1 ppm and reproduced symptoms of plant disease in 70% of the treatments.

The results described above confirm those previously reported concerning the structure–activity relationships of compounds with the botryane skeleton

TABLE 3. FREQUENCY AND SEVERITY OF LESIONS CAUSED BY BOTRYDIAL (**1**) ON *Phaseolus vulgaris* PLANTS

Botrydial concentration (ppm)	Frequency of lesion (%)[a]	Severity of lesion (%)[b]	Time (hr)
1000	100	100	0.5
500	100	100	1
250	100	100	2
100	100	100	2
50	100	40	4
40	100	35	4
30	100	30	6
20	90	15	6
10	80	6	120
1	70	5	120

[a] Arithmetic mean of the results from two independent experiments with 10 leaves for each concentration in each experiment (standard deviation <3%).
[b] Calculated as the surface affected compared to the total surface expressed in mm^2.

(Durán-Patrón et al., 1999). The biological activities of these compounds are related to the oxidation state of the C-10 and C-15 carbon atoms, as well as to the presence of a hydroxyl group on the C-9 carbon atom.

It was also observed that both sunlight radiation intensity and exposure period caused a significant difference in the time necessary for the lesions to appear. Botrydial (**1**), the most active compound, was shown to act rapidly and cause chlorotic lesions in the whole area of application at all the different concentrations assayed when the plant was exposed to bright sun light (514 W/m^2 average radiation; Table 3). In contrast, when the experiment was repeated in dim light (360 W/m^2 average radiation), the same effect took twice as long to occur. At the minimum active concentrations of 10 and 1 ppm, chlorosis took place after 120 hr in 80% and 70% of the treatments, respectively. Other highly active metabolites such as botryendial (**2**) and 8,9-epibotrydial (**4**) showed phytotoxic activity at higher concentrations after 2 and 3 hr, respectively. These experiments were carried out with two genotypes of *Phaseolus vulgaris*: genotype N90598, with well-characterized susceptibility to *B. cinerea*, and genotype N90563, with characterized resistance to the fungus. The results were similar in both cases.

In order to prove the light dependence of botrydial (**1**), the compound was applied at different concentrations (10–150 ppm) to leaves of *Phaseolus vulgaris*, which were then covered with aluminum foil. The leaves were examined every 2 hr for 12 hr and after 24 hr, and no chlorosis was evident. Nevertheless, once these same leaves were exposed to light, chlorosis was observed within 1–2 hr.

In another experiment, botrydial (**1**) was applied to the leaves of *P. vulgaris* at different concentrations, after which the plant was put in bright sunlight (514 W/m^2 average radiation). Once the lesion appeared, the leaf was covered with aluminum foil and the extent of the lesion was noted after 2, 4, 6, and 24 hr. No difference could be observed between the covered lesion and that of a leaf exposed to sunlight over the same time intervals.

As *B. cinerea* is a fungus that can affect all parts of the plant, including the fruit, we decided to observe the effect of this toxin on several fruits. When botrydial (**1**) was assayed on the surface of a fruit, a lesion in the fruit skin was observed in five of the six fruits studied. The exception was green pepper.

Finally, the effect of botrydial (**1**) on fungal penetration was studied on attached leaves of *Phaseolus vulgaris*. Botrydial was applied at different concentrations to the leaves, which were then inoculated with *B. cinerea*. The results showed that botrydial facilitates fungal penetration and colonization of plant tissue. In a typical experiment in which a 250 ppm solution of botrydial was used, 48 hr after the leaves had been inoculated with *B. cinerea*, the fungus had already started to grow, producing a lesion 43 mm in diameter. In contrast, in the inoculation experiment without botrydial, fungal penetration was slower, being visible only after 72 hr, and the diameter of the lesion was only 37 mm.

In conclusion, we have found that *B. cinerea* synthesizes a number of metabolites that reproduce some of the symptoms of the disease caused by this organism. As a result of our experiments, we have also obtained evidence that the action mechanism of the phytotoxin botrydial is light dependent.

Exogenous applications of the most active and abundant metabolite, botrydial (**1**), facilitate fungal penetration and colonization of plant tissue. These results, together with the recent discovery that botrydial is produced in infected plants (Deighton et al., 2001), suggest the involvement of this compound as a toxin in the pathogenesis of *Botrytis cinerea.* Furthermore, the fact that botrydial affects different crops and genotypes seems to indicate that this toxin and its related compounds are nonhost specific toxins that increase the extent of disease, and most likely are virulence factors. Further work is in progress to investigate the mechanistic aspects as well as the scope of the light dependent plant-toxin reaction.

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EFFECT OF PREDATOR DIET ON LIFE HISTORY SHIFTS OF RED-LEGGED FROGS, *Rana aurora*

JOSEPH M. KIESECKER,[1,*] DOUGLAS P. CHIVERS,[2] MICHAEL ANDERSON,[3] and ANDREW R. BLAUSTEIN[4]

[1]*Department of Biology*
The Pennsylvania State University, 208 Mueller Lab
University Park, Pennsylvania 16802-5301

[2]*Department of Biology*
University of Saskatchewan, 112 Science Place
Saskatoon, Saskatchewan, S7N 5E2, Canada

[3]*Department of Biology*
Syracuse University, 108 College Place
Syracuse, New York 13244-1270

[4]*Department of Zoology*
Oregon State University, 3029 Cordley Hall
Corvallis, Oregon 97331

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Abstract—Larval red-legged frogs (*Rana aurora*) are known to exhibit antipredator behavior in response to both chemical alarm cues released from injured conspecifics and chemical cues of predators. In some cases, the response to predators is dependent on the predator's diet. In this experiment, we tested whether long-term exposure to predator chemical cues and alarm cues resulted in alteration of life history characteristics of red-legged frogs. We raised groups of tadpoles in the presence of chemical cues of predators that were either fed conspecifics or heterospecific caddis-fly larvae, chemical cues of injured conspecifics, or a no-cue control. Tadpoles raised in the presence of either a predator fed conspecifics or cues of injured conspecifics metamorphosed earlier and at a smaller size than those exposed to predators fed heterospecifics or the no-cue control. The result suggests that red-legged frogs exhibit a life history shift in response to predatory cues and that this response is dependent on the diet of predators.

Key Words—Alarm cues, amphibians, chemical cues, life history, predation, *Rana aurora*, red-legged frogs, rough-skinned newts, *Taricha granulosa*.

* To whom correspondence should be addressed. E-mail: jmk23@psu.edu

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INTRODUCTION

Interactions among predators, prey, and factors that modify the strength of predator–prey interactions are important for determining the structure of many biological communities (e.g., Menge and Sutherland, 1976; Wellborn et al., 1996). Although many predators can reduce prey abundance, predator effects on prey survival can be modified through a variety of strategies employed by prey. Such strategies include alterations of behavioral responses (e.g., Sih, 1986; Kiesecker et al., 1996; Chivers et al., 1996b), changes in morphology (Appleton and Palmer, 1988; Bronmark and Miner, 1992; McCollum and VanBuskirk, 1996), and shifts in the timing of life-history events (e.g., Dodson and Havel, 1988; Skelly and Werner, 1990; Chivers et al., 1999). Prey that initiate antipredator strategies often have an increased probability of surviving predation events (e.g., Hews, 1988). However, antipredator strategies often take time and energy that would otherwise be available for other activities such as foraging and reproduction (Lima and Dill, 1990). As a result, there should be strong selection on prey to determine vulnerability to predation and to adjust antipredator response accordingly.

The vulnerability of prey to predators may also be influenced indirectly by the presence of heterospecifics that share the same predator. For example, apparent competition describes situations where the increase of one prey species negatively impacts another through increased predation intensity (Vandermeer, 1969; Levine, 1976; Abrams, 1987). Alternatively, increased heterospecific density may have positive effects on prey species when it decreases predation intensity as a result of predator switching (e.g., Murdoch, 1969; Abrams, 1987). In turn, selection should act to either sensitize or desensitize prey to cues of heterospecific predation events, depending on whether the presence of heterospecifics increases or decreases predation intensity.

Many aquatic prey animals, including amphibians, use chemical signals to assess predation risk (e.g., Petranka et al., 1987; Dodson et al., 1994; Kiesecker et al., 1996, 1999; Chivers and Smith, 1998). Cues may arise from the predators or they may be released by prey animals when they are captured by a predator (Chivers and Smith, 1998; Kats and Dill, 1998). However, the response of prey to predators may be context dependent. For example, many prey species only respond to chemical cues of a predator when the predator is fed a diet that contains conspecifics (e.g., Mathis and Smith, 1993; Wilson and Lefcort, 1993; Chivers et al., 1996a). Dietary cues of predators may be an effective way for prey to assess their vulnerability to the predators they encounter (Mathis and Smith, 1993; Wilson and Lefcort, 1993; Chivers et al., 1996; Stabell and Lwin, 1997). For example, Wilson and Lefcort (1993) found that tadpoles of the red-legged frog, *Rana aurora*, exhibit antipredator behaviors in response to predatory rough-skinned newts, *Taricha granulosa*, when newts are fed a diet of conspecifics, but not when newts are fed heterospecifics.

In this study, we raised tadpoles of the red-legged frog in the presence of chemical cues from predatory rough-skinned newts fed conspecifics, newts fed heterospecifics, injured conspecifics only, or a no-cue control. We tested whether tadpoles shifted aspects of the life history in response to potential predation risk. Specifically, we measured whether larval red-legged frog altered the timing of metamorphosis or size at metamorphosis in response to predation risk. While short-term behavioral decisions of prey to predatory chemical cues have been studied extensively, relatively little is known regarding life-history responses of prey to chemical cues that indicate increased predation risk (Chivers and Smith, 1998).

Red-legged frogs and rough-skinned newts can be found together in many of the same breeding habitats where their ranges overlap west of the Cascade–Sierra Nevada mountains from British Columbia, Canada, to northern California, USA (Stebbins, 1985). Rough-skinned newts are effective predators of red-legged frog larvae, although the presence of alternative prey may in some instances lower predation risk for red-legged frogs (Nussbaum et al., 1983; Wilson and Lefcort, 1993). Thus, the presence of newts alone may not accurately predict the level of predation that red-legged frog larvae may expect to experience. We predicted that red-legged frog larvae should only respond to cues of rough-skinned newts when they are associated with chemical cues that give a reliable indication of predation risk experienced by conspecifics.

METHODS AND MATERIALS

We collected *R. aurora* embryos on January 15, 1996, from a pond located 18 km south of Waldport, Oregon, USA. Embryos were collected and transported to a laboratory at Oregon State University where they were reared in 38-liter aquaria. After hatching, tadpoles were raised at approximately 16°C on a 12L:12D photoperiod and fed alfalfa pellets and fish food *ad libitum.* We began tests when all tadpoles reached Gosner (1960) stage 25.

Experiments took place in 24, 38-liter aquaria that were divided widthwise with a fiberglass mesh screen to create two compartments, each measuring 25 × 30 × 25 cm. One compartment of each tank contained a 24-cm-diam. opaque plastic chamber. The side of the chamber facing away from the other compartment was perforated with small (500 μm) holes to allow movement of chemical cues out of the chamber.

We placed 12 randomly selected tadpoles into each of the 24 test tanks, on the side opposite the large plastic chambers. The experiment consisted of four treatments in a randomized block design with six replicates. Treatments 1 and 2 were predator treatments. In treatment 1, tadpoles were exposed to chemical cues of one predatory rough-skinned newt (*T. granulosa*) that was fed caddisfly larvae;

treatment 2 tadpoles were exposed to chemical cues of a predatory *T. granulosa* that was fed conspecifics. Treatment 3 consisted of exposing tadpoles to alarm cues from injured conspecifics. Treatment 4 was a control where tadpoles were exposed to a blank control containing no cues. Within a given treatment, we rotated newts among each of the tanks so that tadpoles were exposed to all newts fed a particular diet.

Throughout the experiment, tanks were cleaned weekly. Newts were fed either three tadpoles or three caddisfly larvae each week. There was always at least one prey item available for each newt. We prepared the alarm cue stimulus by grinding a single tadpole with a mortar and pestle in 60 ml of distilled water. Ten milliliters of the solution was added to each of the six alarm cue treatment containers. Alarm cues were added to the tanks three days per week. On all occasions, the cues were introduced into the plastic chambers. Test tadpoles were fed ground rabbit chow *ad libitum.* All test animals that reached metamorphosis (Gosner stage 41) (Gosner, 1960) were removed from the aquaria, and their mass was determined to the nearest milligram.

Statistical Analysis. For each aquarium, we calculated the mean time tadpoles took to reach metamorphosis and their mean mass at metamorphosis. Because individual responses are not independent of one another, we used tank means for all statistical analyses. We used multivariate analysis of variance (MANOVA) to evaluate the treatment effects on the dependent variables: mean mass at metamorphosis and mean time to metamorphosis (Tabachnick and Fidell, 1989). After MANOVA, we used Bonferroni-adjusted univariate analysis of variance (ANOVA) on each response variable to interpret patterns uncovered by the MANOVA. Tukey (HSD) tests were used to compare treatment means where significant ($P < 0.05$) differences were found with the ANOVA.

RESULTS

The results of MANOVA revealed strong treatment effects on *R. aurora* life-history parameters (Table 1). We used univariate tests (ANOVA) and *post hoc* comparisons (Tukey tests) to interpret patterns of response of time and mass at metamorphosis due to our manipulations.

There were strong treatment effects on the timing of metamorphosis by red-legged frogs (Table 1, Figure 1). They responded to exposure to chemical cues of predatory newts fed conspecifics by initiating metamorphosis more than six days earlier than those exposed to chemical cues of newts fed caddisflies (Table 2, Figure 1). Moreover, frogs exposed to chemical cues of injured conspecifics initiated metamorphosis more than 7 days earlier than those exposed to control conditions (Table 2, Figure 1). Similarly, there were strong treatment effects on frog mass at metamorphosis (Table 1, Figure 1). Frogs exposed to predatory newts

TABLE 1. RESULTS OF MANOVA FOR TREATMENT EFFECTS ON LIFE-HISTORY ATTRIBUTES OF *Rana aurora* (MASS AT AND TIME TO METAMORPHOSIS) AND ANOVAS FOR EACH RESPONSE VARIABLE[a]

MANOVA	$F_{2,19}$	P
Constant	7339.543	<0.001
Treatment	7.423	<0.001
ANOVAs	$F_{3,20}$	P
Mass		
Treatment	13.167	<0.001
Time		
Treatment	9.563	<0.001

[a] Response variables are mass at metamorphosis (mass) and time to metamorphosis (time). Significance level for univariate tests is 0.025 (Bonferroni-adjusted for two response variables).

fed conspecifics were 20% smaller than their counterparts exposed to newts fed caddisflies (Table 2, Figure 1). Additionally, mass at metamorphosis of frogs exposed to chemical cues of injured conspecifics was 14% lower than control animals (Table 2, Figure 1).

DISCUSSION

The results suggest that red-legged frogs alter aspects of their life history in response to chemical cues that indicate predation. Larval frogs initiate metamorphosis earlier and at a smaller size when exposed to cues that denote predation of conspecifics. By decreasing time to metamorphosis, larvae limit their exposure to predatory newts. However, this type of life-history shift likely incurs costs. For example, red-legged frogs that initiate metamorphosis earlier are also smaller. Smaller size at metamorphosis can decrease both reproductive success and survival in the terrestrial environment (e.g., Woodward, 1983). By metamorphosing earlier, frogs also may be exposed to higher levels of terrestrial predation and/or may experience slower growth rates. The cues responsible for initiation of metamorphosis are complex and need further investigation regarding the costs of early metamorphosis.

The experimental design provided insight regarding the specific mechanism responsible for the alterations of life history. Wilson and Lefcort (1993) showed that red-legged frog tadpoles do not initiate antipredator behaviors in response to cues of predatory newts unless newts are fed a diet of conspecifics. In this experiment, we

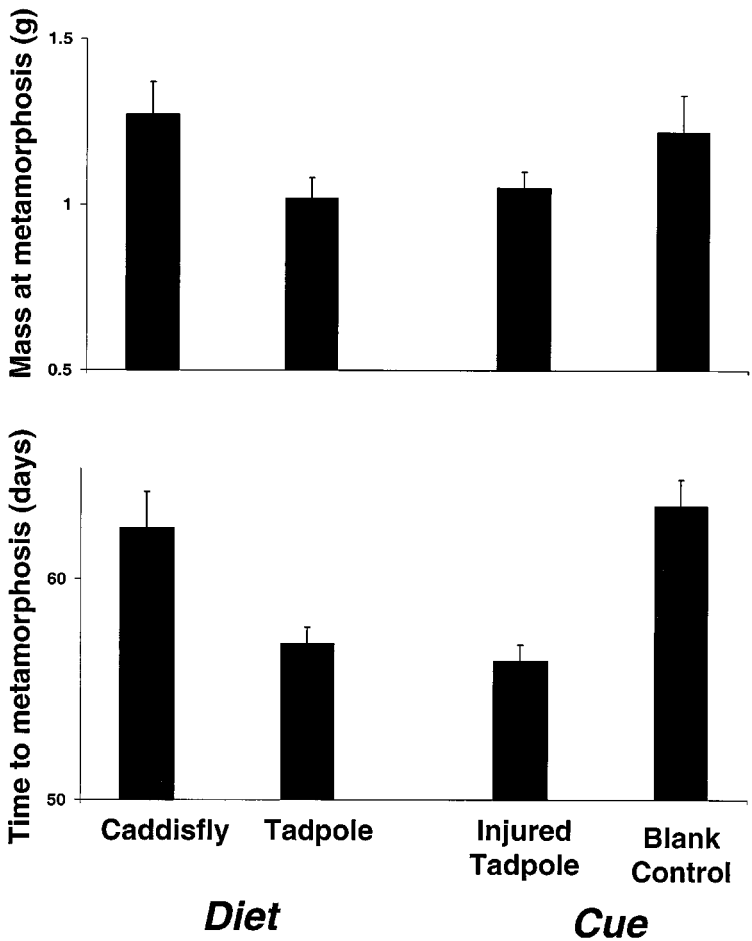


FIG. 1. Mean (+SE) time to metamorphosis (days) and mass at metamorphosis (grams) for *Rana aurora* exposed to chemical cues of predatory newts fed caddisflies, predatory newts fed conspecifics, injured conspecifics, or a blank control.

fed newts tadpoles in experimental chambers. Consequently, the predator stimulus likely resulted in a complex chemical array that included both chemical cues of the predator and injured prey cues. We know that predator stimuli and cues of injured conspecifics alone will induce change in life history. Furthermore, it is clear that exposure to predator cues in the absence of alarm cues will not induce life-history shifts. These results imply that the changes we observed are a direct response to the alarm cues of injured prey and not a reaction to the predator.

TABLE 2. MATRIX OF PAIRWISE COMPARISON PROBABILITIES FOR TUKEY HSD MULTIPLE COMPARISONS[a]

Treatment	1	2	3	4
Mass				
1	1.0	—	—	—
2	<0.001	1.0	—	—
3	0.001	0.961	1.0	—
4	0.728	0.003	0.009	1.0
Time				
1	1.0	—	—	—
2	0.022	1.0	—	—
3	0.007	0.952	1.0	—
4	0.932	0.006	0.002	1.0

[a] Treatment 1 = predatory newts fed caddisflies; treatment 2 = predatory newts fed conspecifics; treatment 3 = injured conspecifics cues; treatment 4 = blank control.

The results parallel those found in other systems. For example, studies of behavioral responses and morphological changes indicate that prey species may only respond to chemical cues of a predator when the predator is fed a diet that contains conspecifics (Mathis and Smith, 1993; Wilson and Lefcort, 1993; Chivers et al., 1996a). The results presented here contribute to a growing body of evidence that suggests prey use chemical alarm cues released from injured conspecifics to alter aspects of their life history. For example, Crowl and Covich (1990) demonstrated that snails, *Physella virgata*, exposed to crayfish feeding on conspecifics were older at first reproduction than *P. virgata* exposed to crayfish not feeding on snails. Moreover, Chivers et al. (1999) showed that western toad tadpoles exposed to chemical cues of injured conspecifics initiated metamorphosis earlier than tadpoles not exposed to injured conspecifics. Future studies should examine the chemical nature of the stimulus that induces the changes (e.g., Wassersug, 1997; Kiesecker et al., 1999), and they should manipulate the timing and frequency of chemical stimuli to assess if alterations of life history can be induced by exposure to stimuli throughout development or whether exposure during certain stages is required to initiate life history shifts.

We expect that initiating antipredator responses by red-legged frog larvae must be costly and only used when predators are focusing their feeding on conspecifics. For example, why would red-legged frogs forgo responding to a potential predator when newts that are feeding on heterospecific caddisflies are still capable of consuming red-legged frog tadpoles? The foraging strategies employed by rough-skinned newts are unknown—whether they focus their feeding on the most abundant prey items or specialize their feeding on particular prey items regardless of relative abundance. However, the lack of response to newts fed heterospecifcs

suggests that the presence of alternative prey such as caddisfly larvae must result in decreased predation intensity on frog larvae. In natural ponds, abundance of heterospecific prey (e.g., caddisflies) varies dramatically between ponds and between years. If heterospecifics offer a release from predation by newts, then red-legged frog tadpoles may not be able to accurately assess predation risk by using cues of newts alone. Thus, using dietary cues of predators, as opposed to using cues directly released from the predators themselves, may provide a more accurate indication of vulnerability.

The ability of prey animals to assess risk and respond flexibly towards different degrees of predation has important ramifications. The failure of a prey animal to recognize and respond to a predator increases the probability that it will be captured during an encounter with a predator. However, a prey animal that gives an antipredator response upon encountering a nonpredator wastes time and energy that would otherwise be available for activities such as foraging and reproduction (Lima and Dill, 1990). Thus, there should be strong selective pressure for prey that can distinguish between predators that pose a risk and those that do not.

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PREY DETECTION BY VOMERONASAL CHEMORECEPTION IN A PLETHODONTID SALAMANDER

JOHN S. PLACYK, JR.[1,2,*] and BRENT M. GRAVES[1]

[1]*Department of Biology*
Northern Michigan University
1401 Presque Isle Ave
Marquette, Michigan 49855

[2]*Department of Ecology and Evolutionary Biology*
University of Tennessee, Knoxville
569 Dabney Hall
Knoxville, Tennessee 37996-1610

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Abstract—While chemoreception is involved in a wide variety of salamander behaviors, the chemosensory system that mediates specific behaviors is rarely known. We investigated the role of the vomeronasal system (VNS) in foraging behavior of the red-backed salamander (*Plethodon cinereus*) by manipulating salamanders' abilities to detect nonvolatile chemical cues emitted by potential prey. Subjects received one of three treatments: (1) impaired vomeronasal system, (2) sham manipulation, and (3) no manipulation. The role of the VNS in mediating foraging on motile prey (*Drosophila melanogaster*) was investigated under three light conditions (bright, dim, dark). Salamanders with impaired VNSs foraged less efficiently than either of the other experimental groups by displaying the longest latency to attack and the lowest rate of prey capture, especially in the absence of visual cues. A second experiment utilized freshly killed prey to determine whether the VNS takes on added importance in the absence of visual or tactile cues associated with moving prey. Animals with impaired VNSs showed a decreased foraging efficiency on stationary prey under both dark and light conditions. In addition, a mark–recapture study of VNS-impaired and sham salamanders in the field also indicated that salamanders with impaired VNSs consumed fewer stationary prey compared to shams. The study indicates that the VNS plays a substantial role in the foraging behavior of the plethodontid salamander, *P. cinereus*.

Key Words—Behavior, chemical senses, foraging, *Plethodon cinereus*, Plethodontidae, salamander, Urodele, vomeronasal system.

* To whom correspondence should be addressed. E-mail: jplacyk@utk.edu

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INTRODUCTION

Terrestrial salamanders are thought to rely primarily on vision while foraging, while olfaction plays a secondary role (Wake and Deban, 2000). However, there are few detailed studies examining the sensory mediation of salamander foraging behavior. Given that the olfactory system of terrestrial salamanders is well-developed and thought to play a role in feeding (Dawley and Crowder, 1995; Wake and Deban, 2000), the current study attempts to elucidate the role of specific olfactory cues in mediating foraging behavior.

The olfactory system of terrestrial salamanders is characterized by an elongate snout that houses paired and cartilaginous olfactory capsules. Within each of these capsules is a large medial olfactory sac, lined with olfactory epithelium [the nasal olfactory system (NOS)], and a small ventrolateral sac, lined with a specialized sensory epithelium [the vomeronasal system (VNS)] (Dawley and Bass, 1989). Most tetrapod vertebrates possess these two separate nasal chemosensory systems. Duvall (1980) described the NOS as being a "quantitative distance-sensing" system that responds to the presence of any pungent substance and initiates further investigation via the VNS, which is more of a "qualitative, or discriminating" system. While the division of function of the two chemosensory systems is not a simple one, the NOS is primarily responsible for the detection of volatile odorants (airborne chemical cues), while the VNS responds primarily to nonvolatile odorants (substrate-borne chemical cues) (Burghardt, 1980; Bertmar, 1981; Halpern, 1987). The functional roles of nasal chemosensory systems have been demonstrated in many tetrapods (reviewed by Halpern, 1992). For example, in lizards, electro-olfactograms (EOG), measures of summed neuronal activity, recorded from the olfactory mucosa in response to airborne odorants are considerably higher than the EOGs elicited by the same odorants from the vomeronasal olfactory epithelium. However, liquid delivery of the same airborne odorants to the vomeronasal olfactory epithelium results in significantly higher EOGs (Halpern, 1992). In our study, we focused on the VNS, by manipulating the ability of a tetrapod to detect and react to chemical cues associated with prey.

The VNS is variable among tetrapods. It ranges from being completely absent or vestigial in birds and some mammals, to being anatomically larger than the main olfactory system in most squamate reptiles (lizards and snakes) (Moulton and Beidler, 1967). In amphibians, the vomeronasal epithelium develops in a ventromedial (anurans) or ventrolateral (urodeles) diverticulum of the nasal cavity, termed the vomeronasal organ (Dawley and Bass, 1988).

Following the highly variable structure of the VNS among tetrapod taxa are diverse behavioral mechanisms by which molecules are transferred to the chemosensory epithelium (Graves, 1993). Mechanisms for chemical delivery to the VNS sensory epithelium include tongue flicking by squamate reptiles (Halpern and Kubie, 1980), flehmen in some mammals (Estes, 1972), and physical pumping of the

vomeronasal organ (Meredith and O'Connell, 1979; Eccles, 1982). Vomeronasal chemoreception among lungless salamanders (plethodontids) is mediated by contact of the nasolabial cirri (fleshy protuberances from the upper lip) to a substrate via "nose-tapping" (Arnold, 1972, 1976). Nonvolatile molecules in aqueous form are then transported from the nasolabial cirri up specialized grooves (the nasolabial grooves) via capillary action to the vomeronasal receptor epithelium in the ventral nasal cavity (Brown, 1968; Dawley and Bass, 1989).

In addition to variability in structure among tetrapods, the VNS also differs in function. In mammals, the primary function is to detect excreted sex hormones and their various breakdown products (Estes, 1972; Halpern, 1987). The VNS of squamate reptiles is responsible for the detection of prey, self, and conspecific cues, as well as for homing (Halpern, 1987; Graves, 1993). While chemical cues are important in the mediation of many salamander behaviors, rarely have researchers investigated the roles of specific chemical senses (i.e., NOS, VNS, and taste).

To investigate the role of the VNS in mediation of salamander behavior, we utilized the red-backed salamander, *Plethodon cinereus*, because the VNS of this species has been implicated in the detection of self, conspecific, prey, alarm cues released by both conspecifics and heterospecifics, and predator cues (Dawley and Crowder, 1995; Wake and Deban, 2000). Plethodontid salamanders are model organisms for studying the VNS, as the system can be easily manipulated by obstructing the flow of nonvolatile molecules up the nasolabial grooves (Dawley and Bass, 1989). However, the role of the VNS in mediation of plethodontid behavior has been directly investigated in only two studies. Graves (1994) found that the VNS is involved in the recognition of home areas by *P. cinereus*, while Jaeger (1981) found that *P. cinereus* that had been bitten on the snout by a conspecific and had at least one nasolabial groove scarred foraged less efficiently on nonmobile prey than *P. cinereus* that had been bitten on the tail.

Plethodon cinereus typically emerge from daytime refugia between dusk and a few hours after dusk, at which time they search the forest floor for prey, relying heavily upon visual cues. Despite these crepuscular tendencies, *P. cinereus* are considered nocturnal (Adler, 1969, 1970) and actively forage in the absence of visual cues (Placyk and Graves, 2001b). In addition, during the day, when less active, they typically seek shelter under logs and leaf litter, which can obscure or eliminate visual cues (Petranka, 1998). Therefore, we hypothesized that this species would rely heavily upon chemical cues, possibly nonvolatile chemical cues detected by the VNS, at night and from under cover objects during the day. David and Jaeger (1981) indicated that salamanders require chemical cues to locate prey in the dark, but did not investigate the role of the VNS specifically. We report here the results of laboratory and field experiments that addressed the questions: (1) What role do chemical cues play in the detection of motile prey under different lighting conditions? (2) What role do chemical cues play in the detection of stationary prey under different lighting conditions? (3) Can the function

of chemical cues in foraging behavior be demonstrated in natural populations of *P. cinereus*?

METHODS AND MATERIALS

General Methods. We collected 50 *P. cinereus* near the town of Big Bay, Powell Township, Marquette County, Michigan, USA. Specimens were placed individually in jars and transported to laboratory facilities at Northern Michigan University. Salamanders were housed individually in Petri dishes (9 cm diam.) lined with damp filter paper, and stored in a controlled temperature chamber at $18 \pm 0.5^{\circ}$C with a 12L:12D photoperiod. Snout–vent length (SVL) was measured to the nearest 0.02 mm with digital calipers, and individuals with SVLs below the estimated minimum adult size (<3.25 cm SVL), (Sayler, 1966) were excluded from experiments. Salamanders were fed *Drosophila melanogaster* (fruit flies) *ad libitum* and permitted to acclimate to laboratory conditions for a minimum of one week before trials were conducted. The acclimation period allowed the salamanders to become experienced with foraging on *Drosophila*. Salamanders that did not eat during the initial one-week acclimation period were not used. Before each experiment, animals were fed to satiation, then not fed again for seven days to provide approximately the same level of hunger for each individual.

Role of Vomeronasal System in Foraging on Motile Prey. The first experiment investigated the role of the VNS in foraging on motile prey, both in the presence and absence of visual cues. Three groups of 15 *P. cinereus* each were used. Thirty animals were anesthetized by placing them in a 30% ethyl alcohol and distilled water solution until they were flaccid when held or did not respond when nudged with a blunt probe (Fellers et al., 1994). Fifteen *P. cinereus* had their nasolabial grooves cauterized under a dissecting microscope with a heated inoculation loop to disrupt delivery of chemicals to the vomeronasal receptor epithelium (Dawley and Bass, 1989; Graves, 1994). Fifteen *P. cinereus* had both cheeks cauterized just posterior to the nasolabial grooves as a sham procedure to control for physical stress associated with the manipulations. The remaining 15 animals were unmanipulated controls. The treatment groups were termed VNX, sham, and control.

Light intensity was manipulated to simulate natural variation in illumination during various parts of the day. Incandescent bulbs of two intensities were used to simulate dim (7-W) and bright (100-W) light that might be experienced in the wild. Both lights were suspended approximately 1.33 m above the test arena to prevent any significant temperature change during the trials. Black light was used to simulate "night-time" conditions. Placyk and Graves (2001a) have shown that *P. cinereus* are unable to detect visual cues under such lighting. The black light was suspended approximately 70 cm above the test arena, as no significant temperature change was expected for this treatment. Temperature in the test arena

was monitored for the duration of each trial. The experimenter was concealed behind a blind to prevent detection of irrelevant visual stimuli. Test subjects were placed in a test arena (a Petri dish with a diameter of 14.5 cm) and permitted to acclimate for 1 min. To eliminate unfamiliar chemical cues, test arenas were washed with distilled water and a clinical detergent powder (Alconox) before each trial. Test arenas were swabbed with filter paper from the home Petri dish of each individual salamander prior to the start of each trial to provide moisture and to deposit familiar chemical cues to minimize escape behavior (Tristram, 1977) and to optimize foraging responses (Jaeger et al., 1981, 1983).

Following the 1-min acclimation period, 15 wingless *D. melanogaster* were added to each Petri dish to induce foraging behavior. Salamanders were tested under the different lighting conditions in random order to counter differences in behavior due to learning (Jaeger and Barnard, 1981; Jaeger and Rubin, 1982; Jaeger et al., 1995). Number of prey captured was recorded during the subsequent 15-min trials. The amount of time subjects spent moving after prey had been introduced to the test arena and latency until the initial attack were recorded. Additional dependent variables quantified included number of both successful and unsuccessful attacks and number of nose taps.

Since much of the data did not meet parametric assumptions, nonparametric statistical analyses were used for comparison purposes. Kruskal-Wallis one-way ANOVAs on ranks, with the three vomeronasal conditions as levels of the independent variable, were used to detect differences within each light condition. Friedman's ANOVAs, with the three light conditions as levels of the independent variable, were used to detect differences within treatment groups. If the Kruskal-Wallis and Friedman's ANOVAs indicated significant differences among treatments, we employed Kruskal-Wallis multiple-comparison Z-value tests and Tukey-Kramer multiple-comparison tests to investigate all pairwise differences, respectively (Roscoe, 1975; Sokal and Rohlf, 1995). Alpha was set at 0.05.

Role of Vomeronasal System in Foraging on Stationary Prey. The second experiment was designed to investigate the role of the VNS while foraging in the absence of visual and tactile cues. Two test groups composed of the 15 sham and 15 VNX individuals from experiment 1 were utilized. The normal salamander group was eliminated, as the data collected in experiment 1 and a previous study (Graves, 1994) indicated that the sham treatment is an appropriate control for the nasolabial groove cauterization treatment. Subjects were tested under dim light and black light. The bright light condition was eliminated, because subjects tested at this light intensity in the first experiment primarily exhibited escape behavior.

The home Petri dishes of each individual salamander were used as test arenas to facilitate concurrent testing of all subjects in each light condition. The larger test arenas did not allow us to test all individuals at once for each light condition. Twenty *D. melanogaster*, killed by freezing for 24 hr and, then thawed

for 1.5 hr, were scattered in each Petri dish (David and Jaeger, 1981). Test subjects were then placed individually into each dish and randomly exposed to one of the two light conditions for 6 hr, during which time the experimenter was not present. At the end of this period, the number of *D. melanogaster* remaining in each Petri dish was recorded.

The data were not normally distributed; therefore, nonparametric statistical analyses were employed. Mann-Whitney U tests were used to compare responses between the two groups within each light condition, and Wilcoxon signed-rank tests were used to compare responses to the two light intensities of individuals that were tested in both conditions (Roscoe, 1975; Sokal and Rohlf, 1995). Alpha was set at 0.05.

Role of Vomeronasal System in Foraging by Free-Ranging Salamanders. The third experiment was conducted at a field site located near the town of Big Bay, Powell Township, Marquette County, Michigan, to investigate the role of the VNS in foraging of free-ranging salamanders. Upon initial collection, *P. cinereus* were taken to the laboratory where they were weighed to the nearest 0.01 g with an electronic balance and measured for SVL to the nearest 0.02 mm. Salamanders were placed in one of two experimental groups of 25 subjects each. One group received the sham treatment, and the other had their VNS impaired by nasolabial groove cauterization as in experiment 1. Salamanders were marked individually by toe-clipping (Martof, 1953; Ott and Scott, 1999). On the day following their capture, subjects were released at the exact location from which they were collected. Surveyors' flags were numbered and placed at the locations to facilitate recapture.

At the end of the one-month trial period, release sites were searched and test subjects were recaptured. Recaptured salamanders were weighed, and their SVLs were recorded. They were euthanized via ventral application of a benzocaine-containing gel (Orajel regular, 7.5% benzocaine) (Chen and Combs, 1999) and placed in 10% formaldehyde after incisions were made in their sides so that the stomach contents would fix rapidly (Jaeger, 1972). Stomach contents were identified to the nearest taxonomic grouping (Bland, 1978), and stomach content volume was calculated with the formula for a cylinder (Jaeger, 1980). To take into account differences in diet due to environmental conditions (Jaeger, 1978, 1990), all salamanders were captured on the same day and collected again on the same day.

Statistical analyses consisted of *t* tests to compare changes in SVLs and weights among the treatment groups over the one-month trial period. In addition, a *t* test was used to compare stomach content volume of shams and VNX subjects. Mann-Whitney U tests were employed when data did not meet parametric assumptions. Chi-square tests of independence were used to compare the frequency of salamanders with particular prey types (sedentary prey, mobile prey) in their stomachs in relation to their treatment group (VNX or sham) (Roscoe, 1975; Sokal and Rohlf, 1995). Alpha was set at 0.05.

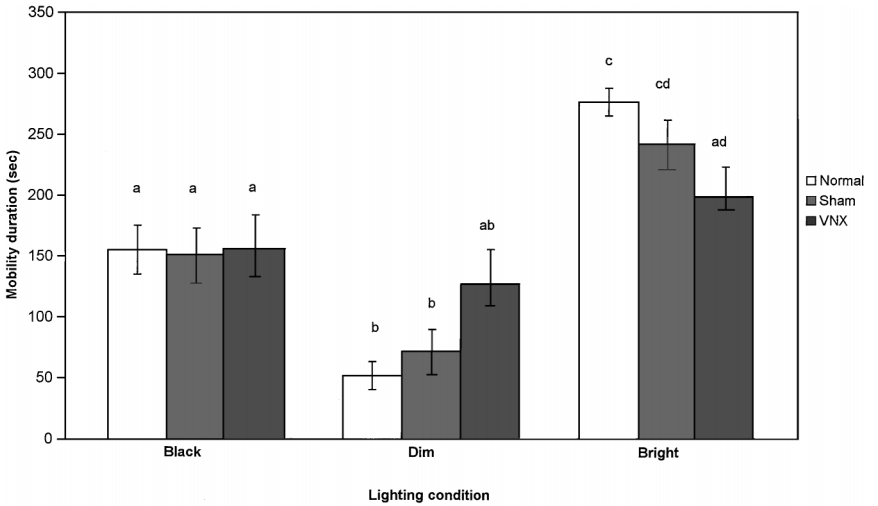


FIG. 1. Mobility duration (mean $\pm$ 1 SE) of normal, sham, and VNX treatment groups with equal sample sizes ($N = 15$) in bright, dim, and black light in the presence of mobile prey. Different letters indicate significantly different means ($P < 0.05$).

RESULTS

Role of Vomeronasal System in Foraging on Motile Prey. In terms of mobility, there was no significant difference in the amount of time moving in normal, sham, and VNX groups in either the black ($H_{(2,44)} = 0.19$; $P = 0.91$) or dim ($H_{(2,44)} = 3.34$; $P = 0.19$) light (Figure 1). However, there was a difference in the amount of time that the three groups spent moving in the bright light ($H_{(2,44)} = 7.81$; $P = 0.02$; Figure 1); VNX subjects moved less than normal individuals ($P < 0.05$), while sham subjects did not differ from either of the other two treatment groups ($P > 0.05$). Within treatment groups, normal ($Q_{(2,14)} = 28.13$; $P < 0.05$), sham ($Q_{(2,14)} = 19.0$; $P < 0.001$), and VNX ($Q_{(2,14)} = 11.29$; $P < 0.05$) salamanders exhibited different amounts of time moving under the three light conditions (Figure 1). Salamanders from the VNX group moved more in bright light than in dim ($P < 0.05$) but not more than in black light ($P > 0.05$). The amount of time the VNX salamanders spent moving in dim light did not differ from black light ($P > 0.05$). Salamanders from the normal and sham groups moved more in bright light than in either dim or black light and more in black light than in dim light ($P < 0.05$).

Latency to first attack differed among the treatment groups in black light ($H_{(2,44)} = 6.40$; $P = 0.04$; Figure 2). Multiple comparison tests showed that VNX salamanders exhibited a longer latency to attack than normal salamanders in black

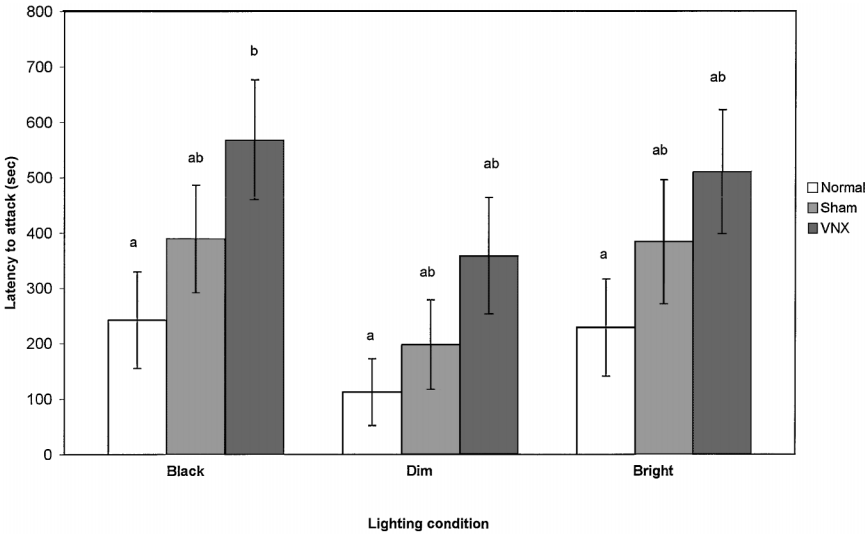


FIG. 2. Latency to attack mobile prey (mean $\pm$ 1 SE) for normal, sham, and VNX treatment groups with equal sample sizes ($N = 15$) in bright, dim, and black light. Different letters indicate significantly different means ($P < 0.05$).

light ($P < 0.05$), but there was no difference between the sham group and either the normal or VNX groups ($P > 0.05$). Conversely, the latency to first attack did not differ significantly among treatment groups in either the dim ($H_{(2,44)} = 2.37$; $P = 0.31$) or bright ($H_{(2,44)} = 2.88$; $P = 0.24$) light (Figure 2). Within treatment groups, there was no difference in latency to first attack under the three light conditions (Figure 2).

Regarding number of attacks, there were significant differences in the number of prey attacked among groups in black light ($H_{(2,44)} = 7.69$; $P = 0.02$), but not in dim ($F_{(2,44)} = 3.60$; $P = 0.16$) or bright ($H_{(2,44)} = 3.06$; $P = 0.22$) light (Figure 3). Specifically, VNX salamanders attacked less prey in black light than normal salamanders ($P < 0.05$), but sham salamanders did not differ in the number of prey they attacked in black light from either normal or VNX salamanders ($P > 0.05$).

The number of attacks on prey by normal ($Q_{(2,14)} = 17.81$; $P < 0.001$) and sham ($Q_{(2,14)} = 12.89$; $P < 0.05$) salamanders differed among light conditions (Figure 3) with each group attacking more prey in dim light than in either bright or black light ($P < 0.05$). However, the number of prey attacked by these two groups did not differ significantly between the bright and black light conditions ($P > 0.05$). There was a marginally significant difference in the number of times VNX salamanders attacked prey under the three light conditions ($Q_{(2,14)} = 4.05$;

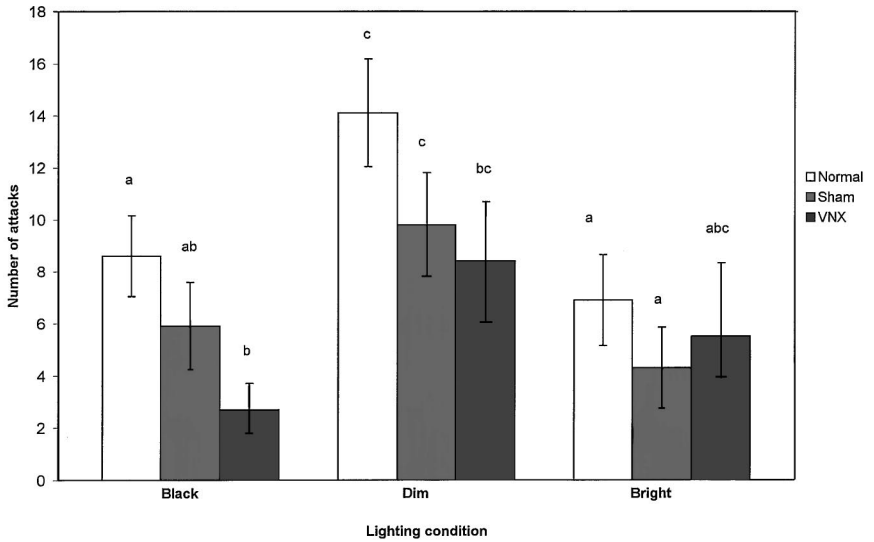


FIG. 3. Number of attacks on mobile prey (mean $\pm$ 1 SE) by normal, sham, and VNX treatment groups with equal sample sizes ($N = 15$) in bright, dim, and black light. Different letters indicate significantly different means ($P < 0.05$).

$P = 0.13$), with fewer prey being attacked in black light than in dim light. However, the number of prey attacked in bright light did not differ from those attacked in either dim or black light.

The number of prey eaten in black light differed among the three groups ($H_{(2,44)} = 6.36$; $P = 0.04$; Figure 4), with the VNX salamanders eating less prey than normal salamanders ($P < 0.05$). However, sham salamanders did not differ in the number of prey they ate under black light from either the normal group or the VNX group ($P > 0.05$). Additionally, the number of prey eaten by the three groups in the dim ($H_{(2,44)} = 3.58$; $P = 0.17$) and bright ($H_{(2,44)} = 2.38$; $P = 0.30$) light did not differ (Figure 4).

Within groups, both normal ($Q_{(2,14)} = 11.92$; $P < 0.05$) and sham ($Q_{(2,14)} = 10.02$; $P < 0.05$) salamanders ate different numbers of prey across the three light conditions (Figure 4), with fewer prey eaten in bright light than in dim light ($P < 0.05$), but no difference was exhibited between black light and the other light conditions ($P > 0.05$). In addition, VNX salamanders also differed in the number of prey they ate across the three light conditions ($Q_{(2,14)} = 8.22$; $P = 0.02$; Figure 4) with fewer prey eaten in bright light than in dim ($P < 0.05$) or black light ($P > 0.05$) and more prey eaten in dim light than in black light ($P < 0.05$).

Regarding nose taps, we found no difference in the number exhibited by groups in either black ($H_{(2,44)} = 0.13$; $P = 0.94$) and dim ($H_{(2,44)} = 4.31$;

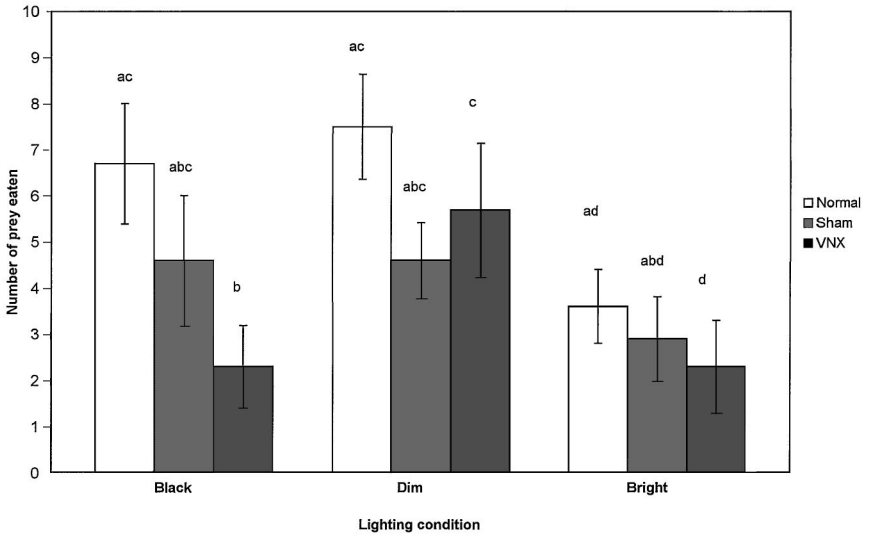


FIG. 4. Number of mobile prey eaten (mean ± 1 SE) by normal, sham, and VNX treatment groups with equal sample sizes ($N = 15$) in bright, dim, and black light. Different letters indicate significantly different means ($P < 0.05$).

$P = 0.11$) light, despite the large numerical difference in mean number of nose taps in the latter (Figure 5). However, the three groups did differ in the number of nose taps they exhibited in bright light ($H_{(2,44)} = 23.74$; $P < 0.001$; Figure 5), with VNX salamanders nose-tapping more than either the sham or normal salamanders ($P < 0.05$). The number of nose taps exhibited by normal salamanders ($Q_{(2,14)} = 12.54$; $P < 0.05$) and by sham salamanders ($Q_{(2,14)} = 9.42$; $P < 0.05$) differed across the light conditions, with each group nose tapping more in black light than in either dim or bright light (Figure 5). Although the VNX group exhibited a higher number of nose taps when compared to the normal and sham groups in all three light conditions, the number of nose taps for this group did not differ among those light conditions ($Q_{(2,14)} = 1.42$; $P = 0.49$; Figure 5).

Role of Vomeronasal System in Foraging on Stationary Prey. We found that a functional vomeronasal system may be important to *P. cinereus* when foraging on stationary prey. In both dim light ($Z = -2.33$, $P = 0.02$; $df = 28$) and black light ($Z = -3.74$; $P < 0.01$; $df = 28$), sham salamanders ate more stationary prey than VNX salamanders (Figure 6). In addition, while sham salamanders did not differ in the number of stationary prey they ate under the two lighting conditions ($Z = 0.8064$, $P = 0.41$, $df = 14$), the VNX salamanders ate less in black light than in dim light ($Z = 2.59$, $P < 0.01$, $df = 14$). Therefore, while visual stimuli coupled with chemical stimuli are important in dim light, a functional vomeronasal

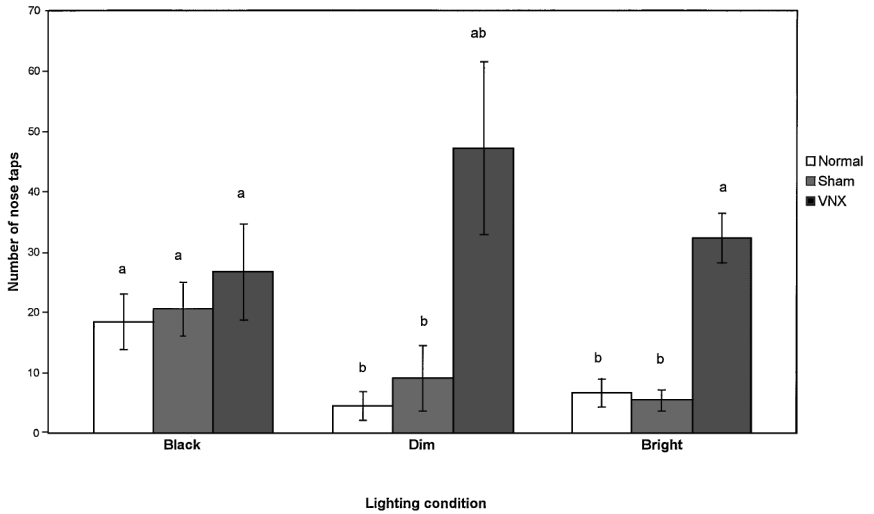


FIG. 5. Number of nose taps (mean $\pm$ 1 SE) exhibited by normal, sham, and VNX treatment groups with equal sample sizes ($N = 15$) in bright, dim, and black light in the presence of mobile prey. Different letters indicate significantly different means ($P < 0.05$).

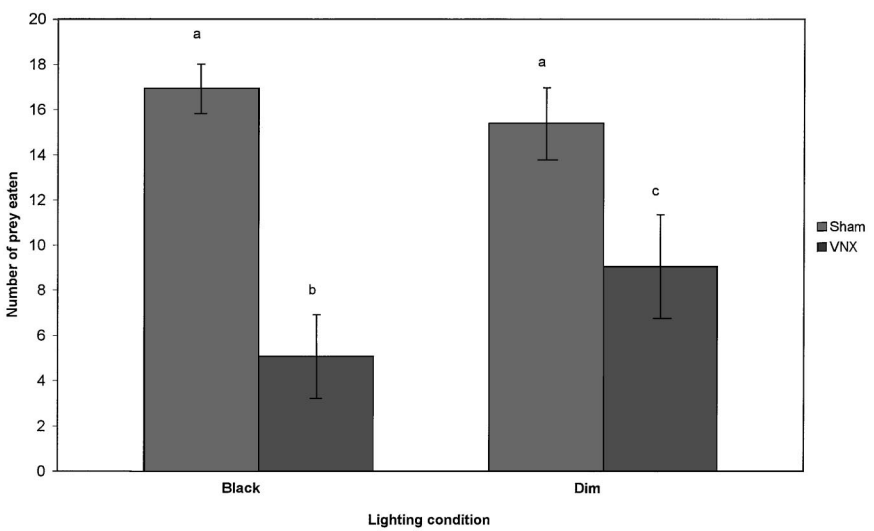


FIG. 6. Number of stationary prey eaten (mean $\pm$ 1 SE) by sham and VNX treatment groups with equal sample sizes ($N = 15$) in dim and black light. Different letters indicate significantly different means ($P < 0.05$).

system is essential for successful foraging on stationary prey in the absence of visual stimuli.

Role of Vomeronasal System in Foraging by Free-Ranging Salamanders. When examining the change in SVL ± SE (millimeters) among the groups over the one-month period, sham salamanders (1.7 ± 1.675) showed a marginally significant increase in SVL, while VNX salamanders (-2.44 ± 1.42) did not ($t = 1.897$; $P = 0.066$ $df = 37$). There was no difference in the change in weight ± SE (grams) ($t = 1.417$; $P = 0.165$ $df = 37$) between the sham (0.12 ± 0.105) and the VNX (-0.073 ± 0.089) group over the one-month test period. In addition, there was no difference between stomach content volumes (cubic millimeters) of the sham (28.69 ± 5.99) and the VNX (36.91 ± 10.91) groups ($U = 171.00$; $P = 0.612$; $df = 37$). However, upon comparing the stomach contents of each group, we found qualitative differences in prey composition and differences in the frequency of salamanders eating specific types of prey.

Arachnids (22%), coleopterans (23%), and arthropod eggs (21%) accounted for the majority of the 58 identifiable prey items found in the 18 recaptured sham salamanders' stomachs (Table 1). Coleopterans (26%), dipterans (14%), and arachnids (12%) comprised over 50% of the 83 identifiable prey items from the 21 recaptured VNX salamanders' stomachs, with arthropod eggs comprising a conspicuously low percentage of the total diet (1%) (Table 1). A χ^2 test of independence indicates that there is a relationship between the frequency of salamanders with stationary prey in their stomachs and treatment group ($\chi^2 = 5.39$; $P < 0.025$; $df = 1$), with sham salamanders (33%) eating more stationary prey than VNX salamanders (4%). However, there was no relationship between the frequency of salamanders with mobile prey items in their diet and treatment group, as 100% of the salamanders that had food in their stomachs ate mobile prey, regardless of treatment group.

DISCUSSION

Role of Chemical and Visual Cues in Detection of Motile Prey. We found that normal and sham animals moved significantly more in black light than in dim light, suggesting the combination of visual and chemical cues in dim light allowed them to use an ambush foraging strategy (remaining stationary waiting for prey to approach) (Placyk and Graves, 2001b). Conversely, VNX salamanders moved more than either the normal or the sham group in dim light, suggesting that impairment of the VNS disrupts this ambush foraging strategy in this light. In addition, all treatment groups moved more in bright light. This greater mobility is most likely associated with an aversion to bright light, as *P. cinereus* are adapted for life under the canopy, fallen logs, and leaf litter of secondary deciduous forests, where there is little or no direct light (Petranka, 1998). Furthermore, if bright light

TABLE 1. STOMACH CONTENTS OF FREE-RANGING SHAM-TREATED ($N = 18$) AND VNX ($N = 21$) *Plethodon cinereus*[a]

Prey item (phylum, order, family)	Occurrence		Percent of total	
	In sham stomachs	In VNX stomachs	For sham group	For VNX group
Annelida				
Arthropoda				
Coleoptera				
Oligochaeta	3	10	5	12
Carabidae	1	3	2	4
Curculionidae	10	16	17	19
Pentatomidae	1	0	2	0
Phalacridae	0	1	0	1
Pselaphidae	0	1	0	1
Staphylinidae	1	1	2	1
Collembola				
Poduridae	1	1	2	1
Diptera				
Drosophilidae	0	1	0	1
Muscidae larvae	0	9	0	11
Simuliidae	3	2	5	2
Hymenoptera				
Cynipidae	0	1	0	1
Formicidae	5	3	9	4
Ichneumonidae	0	3	0	4
Lepidoptera				
Geometridae larvae	1	0	2	0
Pyralidae larvae	1	4	2	5
Neuroptera				
Myrmeleontidae larvae	0	1	0	1
Thysanura				
Machilidae	0	1	0	1
Other arthropods				
Arachnida	13	10	22	12
Diplopoda	2	1	3	1
Eggs	12	1	21	1
Mollusca				
Gastropoda	3	5	5	6
Nematoda	1	8	2	10
Total	58	83		

[a] Prey items are ordered alphabetically starting at the phylum level.

increases the visibility of *P. cinereus* to predators and the chances of desiccation, it may be optimal for salamanders' fitness to avoid such conditions. Ducey and Brodie (1983) have shown that plethodontids will exhibit elevated levels of mobility when the threat of being preyed upon increases. However, none of this evidence explains

why normal salamanders moved more in bright light than the VNX treatment group. We postulate that this increase in mobility is either a spurious effect or has a biological interpretation that is not apparent.

Latency to first attack by normal, sham, and VNX salamanders did not differ among groups in dim or bright light. This suggests that VNX animals were able to locate and attack prey under dim light as quickly as the normal group, although VNX salamanders exhibited an increase in mobility to achieve this equivalent latency time. Conversely, Graves and Duvall (1985a) found that avomic prairie rattlesnakes, *Crotalus v. viridis*, never struck, located, or swallowed prey, whereas unimpaired subjects did. Vomeronasal-impaired *P. cinereus* from our experiment foraged on motile prey when they were available. Apparently, the loss of vomeronasal chemoreception in some species can be compensated for if alternative cues are available (Kardong, 1992; Graves et al., 1993). Under black light, latency to first attack was greater for VNX salamanders than for normal salamanders. This suggests that *P. cinereus* employ the VNS for prey location in the dark, but other sensory systems, such as nasal olfactory, taste, or tactile systems, may be used as well. Overall, the greatest mean latency to first attack across all nine light and salamander treatment combinations was exhibited by the VNX group under black light. This indicates that the VNS plays a role in initial prey detection by salamanders in the absence of visual stimuli.

Vomeronasal-impaired salamanders attacked and consumed fewer prey than normal salamanders in black light, but the difference was absent under dim or bright light. This indicates that a functional VNS is important to *P. cinereus* when foraging in the absence of visual cues, but when visual cues are available, the VNS plays a secondary role. In addition, since visual cues are available in dim and bright light, and since the chemical senses probably play no role in the consumption of prey once detected, one would expect any dependence on the VNS while foraging in lighted conditions to be minimal. The fact that there is no difference between the number of attacks and the number of prey consumed by the three groups in dim and bright light is contradictory to work that has been conducted with squamate reptiles. For example, both scincid lizards and the rattlesnake, *C. v. oreganus*, with impaired VNSs attacked but never swallowed the prey (Graves and Halpern, 1990; Alving and Kardong, 1996), and as described above, avomic *C. v. viridis* never attacked prey (Graves and Duvall, 1985a). These differences in foraging behaviors exhibited by avomic animals may be a result of the methodology used to impair the VNS in each experiment. The vomeronasal ducts that lead to the vomeronasal organ of both the scincid lizards (Graves and Halpern, 1990) and *C. v. viridis* (Graves and Duvall, 1985a) were either sealed with glue or sutured shut, thus preventing access of molecules to the vomeronasal receptors. *C. v. oreganus* were rendered avomic via bilateral lesions of the vomeronasal nerves (Alving and Kardong, 1996).

It is possible that chemical cues could have been transferred to the vomeronasal organ of the salamanders in our experiment if they entered the

nasolabial grooves above the cauterized portion of the groove. In addition, since the VNS of amphibians is located in a ventral pouch of the nasal cavity (Dawley and Bass, 1988), molecules may also reach the vomeronasal receptors from the throat via the internal nares. Therefore, our VNX salamanders may have still had partial use of their VNS (Dawley and Bass, 1989). It is also possible that the scincid lizards and rattlesnakes are more reliant upon chemical cues to differentiate between prey and nonprey, whereas *P. cinereus* may utilize a combination of sensory modalities when choosing appropriate prey.

Vomeronasal-impaired *P. cinereus* did not differ in the number of nose taps they exhibited across the three light conditions, but they nose-tapped significantly more in bright light than either normal or sham animals. Normal and sham salamanders did not differ from each other in the number of nose taps they exhibited in any light, but both nose-tapped more in black light than in either of the other two light conditions. This suggests an increase in chemosensory behavior in the presence of motile prey in the dark. Red spitting cobras (*Naja mossambica pallida*) exhibit increases in a chemosensory behavior (tongue-flicking) when visual cues are eliminated via blindfolds (Kardong et al., 1997). In addition, increases in chemosensory behavior coupled with high mobility, as seen with our salamanders in black light, have been demonstrated in studies conducted with lizards, suggesting that animals employing active foraging strategies (moving through the habitat searching for prey) rely primarily on chemical cues to find prey (Huey and Pianka, 1981; Cooper, 1994a,b, 1995). These results and those of Placyk and Graves (2001b) suggest that, in general, there may be an association between activity level and nose-tapping in plethodontid salamanders, whether visual stimuli are present or absent. The hypothesis that activity and chemosensory behavior covary, however, was not explicitly tested in our current experiments and requires more support.

Vomeronasal-impaired salamanders nose-tapped more than normal or sham animals under dim and bright light, but not under black light. The rate of nose-tapping by sham and normal salamanders in black light was similar to the rate exhibited by the VNX salamanders in all light conditions.

Impairment of the VNS resulted in an increased number of nose taps in the presence of motile prey in dim light (Figure 5). Since the ability to detect nonvolatile chemical cues is eliminated through cauterization of the nasolabial grooves (Dawley and Bass 1989), this increase in nose taps may be a futile attempt to illicit a chemosensory response from the vomeronasal system. In prairie rattlesnakes, mouth gaping and head shaking are believed to facilitate vomeronasal olfaction (Graves and Duvall, 1983). Graves and Duvall (1985b) found that VNX prairie rattlesnakes exhibited greater numbers of mouth gapes and head shakes than sham subjects when exposed to prey. The increase in nose taps among VNX salamanders may indicate that the animals did not exhibit a foraging response due to the lack of chemical signals, but behaved as they would in the absence of prey chemical cues. For example, Placyk (2000) found that, in the absence of prey, normal

salamanders exhibited a greater number of nose taps than when prey were present. Therefore, VNX salamanders that lack the ability to detect prey chemical cues efficiently may not express the foraging behavior typical of a salamander with an unmanipulated VNS.

In summary, the vomeronasal-impaired salamanders were not as effective at foraging on motile prey as salamanders with intact VNSs, especially in the absence of visual stimuli. Vomeronasal-impaired salamanders showed increased mobility under dim light, whereas control groups maintained an ambush foraging strategy. In addition, VNX salamanders exhibited an increase in latency to first attack and a decrease in both the number of attacks and the number of prey eaten in the absence of visual stimuli. Therefore, depending on environmental conditions, chemical cues are important for successful foraging by *P. cinereus*, and the results suggest that the VNS plays a significant role in the detection of those cues.

Role of Chemical and Visual Cues in Detection of Stationary Prey. Comparisons across light conditions showed that VNX salamanders consumed significantly less prey in black light than in dim light, while sham salamanders did not differ in this regard. Thus, the availability of visual cues influenced the successful capture of nonmotile prey items. In addition, comparisons across treatment groups showed that VNX animals ate less than sham salamanders in both dim and black light. This indicates that the VNS plays a role in foraging on stationary prey items. It has been shown previously that *P. cinereus* with at least one nasolabial groove damaged via aggressive confrontations with conspecifics forage less effectively on nonmotile prey (Jaeger, 1981). While this study provides a more systematic approach at attempting to impair the VNS, the results of Jaeger (1981) suggest that even minimal damage to the nasolabial grooves decreases the foraging abilities of *P. cinereus*. Regardless, it appears as though plethodontid salamanders forage more efficiently when multiple sensory systems are employed, as our results indicate that a combination of both visual and chemical cues resulted in the greatest foraging efficiency. This reliance on multiple sensory systems for foraging effectively has also been indicated in two other caudates (*Notophthalmus viridescens* and *Ambystoma tigrinum*), which require a combination of visual and chemical cues to exhibit high rates of foraging behavior (Martin et al., 1974; Lindquist and Bachmann, 1982).

Can Function of Chemical Cues in Foraging Behavior be Demonstrated in Natural Populations of Plethodon cinereus? A significant difference in the types of prey eaten by our two treatment groups was evident. The majority of identifiable prey items removed from the stomachs of sham salamanders were beetles, arachnids, and arthropod eggs. In comparison, beetles, flies, and arachnids comprised the highest percentage of identifiable prey removed from VNX salamander stomachs. Vomeronasal-impaired salamanders had only 1% of their identifiable stomach contents consisting of arthropod eggs, and this difference from the amount of arthropod eggs found in sham salamanders' stomachs was significant. Thus, a functional vomeronasal system might be important for successful foraging on

particular types of stationary prey items, as demonstrated in both our laboratory and field studies. Despite consuming few stationary prey, VNX animals compensated for this inability by taking more motile prey than salamanders with intact vomeronasal systems. This is indicated by the lack of difference between the stomach content volume of the two groups.

In spite of the differences in diet composition, VNX and sham groups did not show a significant change in SVL or weight over the one-month period. This contradicts Jaeger's (1981) study on free-ranging *P. cinereus*, which indicated that individuals who showed damage to one or both of their nasolabial grooves exhibited lower weight–length relationships than those that were uninjured. However, Jaeger (1981) failed to take into account that low body weights may have been the cause of nasolabial groove damage, rather than the result, as individuals with low weight–length ratios may have a high probability of losing fights and thus incurring nasolabial groove damage. Similar to our study, Graves et al. (1993) found that sham and VNX garter snakes, *Thamnophis sirtalis*, collected again after a three-week period, showed no significant difference in change in body mass change.

Implications for Natural Populations. Plethodon cinereus suffer damage to their nasolabial grooves as a result of fighting on a regular basis under natural conditions (Jaeger, 1981), with 11.8% of wild-caught adults having scar tissue on their nasolabial grooves. Jaeger et al. (1982), in a study of intraspecific aggression among *P. cinereus*, also showed that more bites were directed at the snout than at the tail. Our experiments provide empirical evidence that disruption of the vomeronasal system significantly decreased the ability of *P. cinereus* to forage on prey in the absence of visual cues, regardless of their initial weight/length relationships. It is possible that the decreased foraging efficiency exhibited by salamanders with damaged nasolabial grooves may result in a decrease in the fitness of those individuals, but such long-term effects could not be demonstrated over the relatively short period of time in which we conducted our studies.

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EVALUATION OF SYNTHETIC HYDROCARBONS FOR MARK–RECAPTURE STUDIES ON THE RED MILKWEED BEETLE

MATTHEW D. GINZEL* and LAWRENCE M. HANKS

Department of Entomology
University of Illinois at Urbana-Champaign
Urbana, Illinois 61801

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Abstract—This study evaluates the potential for using blends of synthetic hydrocarbons in mark–recapture studies of insects. To test the durability of hydrocarbons, we applied a blend of five straight-chain hydrocarbons (C_{24}, C_{25}, C_{26}, C_{28}, C_{30}) to detached elytra of the red milkweed beetle, *Tetraopes tetrophthalmus* (Forster) (Coleoptera: Cerambycidae), mounted the elytra on pins, and placed them in an exposed location outdoors. The amount of hydrocarbons on the elytra did not change over time, even after two months of exposure to sun and rain. Synthetic hydrocarbons applied to the elytra of living beetles did not significantly influence their longevity or mating success in a laboratory study, and the amounts of hydrocarbons did not change with age. The invariability of hydrocarbon ratios over time suggests that blends could provide a nearly infinite variety of ratios to mark individual insects uniquely and indelibly with a hydrocarbon "fingerprint." This technique offers a convenient, safe, and durable means of individually marking insects and may find application in field studies of larger bodied insects that are long-lived and sedentary.

Key Words—Dispersal, marking techniques, population dynamics, cuticular hydrocarbons, Cerambycidae, *Tetraopes tetrophthalmus.*

INTRODUCTION

Mark–recapture techniques are commonly used to estimate population densities of animals and study their dispersal behavior (Hagler and Jackson, 2001). For this research, it is essential that markings be easy to apply and detect, be durable, and have no adverse effect on the development, behavior, or longevity of subjects

* To whom correspondence should be addressed. E-mail: ginzel@uiuc.edu

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(Southwood and Henderson, 2000). A variety of effective marking techniques have been developed, each with its own drawbacks. For example, materials most often used for marking insects (paints, dyes, physical tags) are easily detected, but can fade, rub off, or dislodge, and may inhibit movement of small insects or render subjects conspicuous to vertebrate predators (Southwood and Henderson, 2000; Hagler and Jackson, 2001). Mutilation marking for insects would involve clipping appendages or damaging the cuticle and would be appropriate only for heavily sclerotized insects or those with large wings (Hagler and Jackson, 2001). Application of many of these markers involves handling the insects, which subsequently may influence their dispersal behavior (Singer and Wedlake, 1981).

Fluorescent dyes can be applied as dusts to groups of insects (Crumpacker, 1974), but may have sublethal effects (Labrecque, 1975). Radioactive isotopes (e.g., ^{14}C and ^{65}Zn) were once popular for marking insects (Service, 1993) but have been replaced by trace elements of low toxicity, such as rubidium, that also have the advantage of persisting in tissues (Hagler and Jackson, 2001). Rubidium levels may rapidly decline, however, once the marked insect is removed from the Rb-containing host (Fleischer et al., 1986), and high levels of exposure can affect developmental rate of larvae and kill adults (Stimmann, 1973; Culin, 1986). These rare elements are also inconvenient markers because they require extensive sample preparation, and detection by rather costly atomic absorption spectrophotometry (Hagler and Jackson, 1998). Immunoproteins can be used to mark groups of small insects, but also involve relatively complicated laboratory assays and are rapidly degraded by rain or high humidity, limiting their utility for some field studies (Hagler and Jackson, 1998).

Our study examines the potential for using blends of synthetic long-chain hydrocarbons, which are structurally stable and nonvolatile, to mark insects for field research. Long-chain hydrocarbons occur naturally in the cuticular wax layer of insects and serve as contact pheromones in some species (Howard and Blomquist, 1982). We tested the durability of hydrocarbon markers with a field study, and also tested for negative affects on the mating success and longevity of our model insect, the red milkweed beetle, *Tetraopes tetrophthalmus* (Forster) (Coleoptera: Cerambycidae). This beetle is endemic to North America and common on its milkweed hosts (Yanega, 1996).

METHODS AND MATERIALS

We prepared a blend of synthetic hydrocarbons (Aldrich, Milwaukee, Wisconsin, USA) by mixing stock solutions of 0.05 g of tetracosane (C_{24}), pentacosane (C_{25}), hexacosane (C_{26}), octacosane (C_{28}), and triacontane (C_{30}) in 1 ml of hexane, and combining 20-μl aliquots of each standard in a 4:2:1:2:4 ratio, respectively. Preliminary analysis of hydrocarbon profiles of *T. tetrophthalmus* of both

sexes by gas chromatography – mass spectrometry revealed there were no major peaks that overlapped with those of the synthetic compounds.

To test the durability of hydrocarbon markers, we removed elytra from field-collected *T. tetrophthalmus* (sex not determined), mounted them individually on insect pins, and arrayed the pinned elytra on a square corkboard (30 cm^2). Half of the elytra were treated with 2 μl of hydrocarbon solution ($N = 25$), and the other half with 2 μl of hexane as a control ($N = 25$). The board was then placed flat on the rooftop of our laboratory building where it was exposed to full sunlight and weather. The board was left in place for 55 days between June 11 and August 5, 2000, during which time daily maximum temperatures averaged $29 \pm 3^{\circ}$C; there were 15 cloudless days, and 17.3 cm of rainfall (Illinois State Water Survey, Department of Natural Resources, University of Illinois at Urbana-Champaign).

We extracted hydrocarbons from the elytra after 0, 1, 7, 30, and 55 days ($N = 5$ elytra per date and treatment) by dipping them in 0.5 ml of hexane for 5 min (see Millar and Sims, 1998). We spiked extracts with 2 μl of a 0.01g/ml docosane solution as an internal standard (docosane does not naturally occur on the cuticle of *T. tetrophthalmus*). We quantified amounts of compounds (as fractions of the amount of internal standard) with a Hewlett-Packard 5973 mass spectrometer interfaced with a HP 6890 gas chromatograph, with an HP-5MS (cross-linked 5% phenyl-methyl siloxane) capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) in splitless mode, with helium as the carrier gas. The injection port was maintained at 250°C, and the oven temperature was ramped from 200 to 240°C at 5°C/min, then 240 to 250°C at 2°C/min. We tested for changes over time in the total amount of synthetic hydrocarbons (the five compounds combined) by one-way analysis of variance (ANOVA; SAS Institute, 2001) with day being the main effect, and tested for changes in relative proportions of individual compounds by two-way ANOVA with main effects being date and compound.

To determine whether our blend of synthetic hydrocarbons influenced longevity and mating success of *T. tetrophthalmus*, we placed six females and four males (collected from the field) in each of five aluminum screen cages (30 cm on a side), and applied 2 μl of hydrocarbon blend to the elytra of half of each sex, and 2 μl of hexane to the other half. To indicate treatments, we marked beetles with a dot of enamel paint of different colors (Testors, Phoenix Model Co., Brooksville, Florida, USA). In each cage, we provided a fresh milkweed umbel and foliage in water for food; cages were kept in the laboratory. We made hourly observations between 08:00 and 17:00 hr for as long as beetles lived to measure mating success (number of times mating) and longevity. In the field, *T. tetrophthalmus* are active from early morning to late afternoon (personal observations), and males and females may remain in copula for more than an hour (McCauley, 1982). The influence of hydrocarbon marking on mating success and longevity was tested by two-way ANOVA with main effects being chemical treatment and beetle sex, and cage as a random effect. To evaluate the durability of hydrocarbons on living beetles, we

extracted the hydrocarbon-marked beetles after they died, quantified amounts of hydrocarbons (as described above for elytra), and tested for changes in total amount of hydrocarbon with beetle age (time since marking) using one-way ANOVA.

We also determined whether *T. tetrophthalmus* was affected by the solvent hexane, by using an experiment of identical design to the above, but applying 2 μl of hexane to the elytra of half of the beetles of each sex, leaving the other half as controls (four cages per treatment), and testing the treatment effects on mating success and longevity by ANOVA. We present means $\pm$ 1 SE throughout.

RESULTS

The total amount of synthetic hydrocarbons (all five compounds combined) on the pinned beetle elytra declined by only ~25% over the two month period (0.12 ± 0.067, 0.11 ± 0.028, 0.11 ± 0.023, 0.079 ± 0.013 and 0.089 ± 0.00025 μg for 0, 1, 7, 30, and 55 days, respectively), but these differences were not statistically significant (ANOVA $F_{4,19} = 0.33,\ P > 0.86$). In addition, the relative proportions of the individual compounds were consistent over time, as indicated by the insignificant interaction term in the ANOVA ($F_{16,24} = 0.24,\ P = 0.99$; time effect $F_{4,24} = 1.19,\ P = 0.32$; compound effect $F_{4,24} = 9.44,\ P < 0.001$; Figure 1).

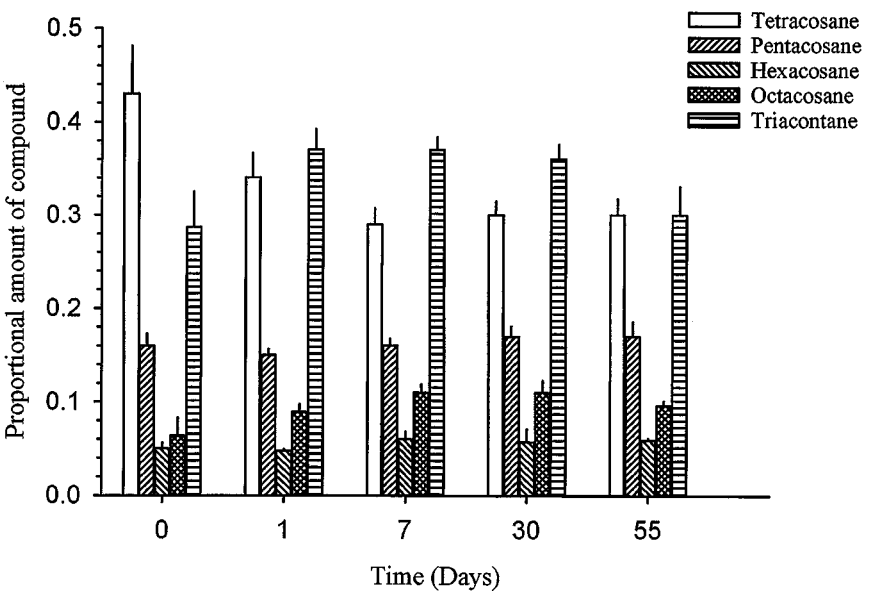


FIG. 1. Proportion of individual hydrocarbons to the total extracted from elytra of *T. tetrophthalmus* that had been exposed outdoors.

Male *T. tetrophthalmus* were observed to mate 0–18 times (average 5.9 ± 1.33), and females mated 0–13 times (average 4.44 ± 0.63) while caged in the laboratory, and the number of matings was not influenced by hydrocarbon treatment (ANOVA treatment effect $F_{1,40} = 0.23,\ P = 0.64$). Moreover, hydrocarbon treatment did not influence longevity: hydrocarbon- and hexane-treated beetles lived 12.7 ± 1.32 and 14.1 ± 1.40 days, respectively (ANOVA $F_{1,33} = 0.65,\ P = 0.43$). The total amount of synthetic hydrocarbons on marked beetles did not vary with their age, averaging 0.0365 ± 0.017 μg across treatments (day effect not significant; ANOVA $F_{6,13} = 0.94,\ P = 0.52$). As with the hydrocarbon-marked elytra, the relative proportion of compounds (individual peaks divided by the total) applied to living beetles did not vary among time periods of 0–4 days, 5–8 days, and 9 and 15 days (ANOVA interaction term $F_{8,14} = 0.68,\ P = 0.71$; time effect $F_{2,14} = 1.34,\ P = 0.27$; compound effect $F_{4,14} = 13.32,\ P < 0.001$).

Hexane alone did not affect mating success of *T. tetrophthalmus*, with hexane-treated and untreated beetles mating an average of 3.76 ± 0.86 and 3.12 ± 0.68 times, respectively (means not significantly different, treatment effect $F_{1,43} = 0.39,\ P = 0.53$). Solvent did not influence longevity: hexane-treated and untreated beetles lived 9.1 ± 1.34 and 9.9 ± 1.42 days, respectively (means not significantly different, treatment ANOVA $F_{1,32} = 0.15,\ P = 0.70$).

DISCUSSION

The amounts of synthetic hydrocarbons that we had applied to beetle elytra changed little over time, even after nearly two months of exposure to high temperatures, sunlight, and rain. This invariability of hydrocarbon ratios over time suggests that blends would provide a nearly infinite variety of ratios that could be used to uniquely and indelibly mark individual insects with a synthetic hydrocarbon "fingerprint." Hydrocarbon blends are easily applied in the field with a small paintbrush or can be applied to some insects by spraying; this labeling is unlikely to influence rates of predation. It takes only a few minutes to extract hydrocarbons from each insect, and analysis requires ~15 min per sample by gas chromatography. Hydrocarbon markers could also be quickly and easily detected, without harming marked insects, by solid-phase microextraction (Shirey, 1999). In a preliminary study, we detected synthetic hydrocarbon blends from the marked elytra of dead *T. tetrophthalmus* using a 7-μm polydimethylsiloxane fiber designed to extract high molecular weight compounds (Supelco, Bellefonte, Pennsylvania, USA). Finally, the synthetic hydrocarbons we used for marking are relatively inexpensive: a kit of straight-chain alkanes (C_5–C_{30}) costs ~US$200 (Aldrich, Milwaukee, Wisconsin, USA) and would yield more than 10 liters of solution per compound, enough for a career of mark–recapture experiments.

Our study confirmed that synthetic hydrocarbons applied directly to the elytra of *T. tetrophthalmus* did not adversely affect mating success or longevity;

however, sublethal effects of hydrocarbons or solvent may influence, for example, dispersal or reproduction. In particular, this technique is probably not suitable for marking soft-bodied insects because hexane may be more readily absorbed through their cuticles. Because cuticular hydrocarbons may play a role in chemical communication among insects (Howard and Blomquist, 1982), care also must be taken in selecting synthetic hydrocarbons that will not influence mating behavior and other intraspecific interactions. Marking insects with hydrocarbons that do not naturally occur on the cuticle, however, is unlikely to influence mating behavior.

We conclude that application of blends of synthetic hydrocarbons offers an effective, convenient, and inexpensive means of uniquely marking insects for long-term field studies of population size and dispersal, and is probably best suited for marking long-lived, large bodied, and heavily sclerotized insects, such as orthopteroids, coleopterans, hymenopterans, and lepidopterans. Because detection of the marker requires chemical analysis, the technique is probably best suited for relatively sedentary insects that would be recaptured at a greater rate and so would require smaller sample sizes. The technique could be extended by using as markers branched-chained hydrocarbons to provide a greater selection of synthetic markers or halogenated hydrocarbons that can be detected with greater sensitivity by electron-capture gas chromatography.

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EVIDENCE FOR A SEX PHEROMONE IN BARK BEETLE PARASITOID *Roptrocerus xylophagorum*

BRIAN T. SULLIVAN*

USDA Forest Service
Southern Research Station
2500 Shreveport Hwy
Pineville, Louisiana 71360

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Abstract—Male *Roptrocerus xylophagorum* (Ratzeburg) (Hymenoptera: Pteromalidae) exhibited courtship and mating behaviors including wing fanning, antennation, mounting, and copulation attempts when exposed to glass bulb decoys coated with a whole-body extract of females in hexane, acetone, or methanol. Activity of extract-treated decoys declined gradually over one week. Males responded much less strongly to freeze-killed female cadavers extracted with solvents than to unextracted cadavers; treatment of extracted cadavers with female extract restored male responses. The pheromone was found to be equally present over the surface of both the abdomen and head/thorax of females, and the origin of the pheromone could not be conclusively localized to any single body region. The activity of pheromone on females increased between day 1 and days 3–5 following eclosion; otherwise, pheromone activity was not significantly affected by either female age or mating. Males were arrested within the zone of a glass surface on which females had walked, suggesting that the pheromone might be substrate-borne. Recent exposure to females reduced male responsiveness, but responsiveness was fully restored after a few hours of male isolation from females. When hexane extracts of whole females were fractionated on silica gel, the pheromone's activity was largely recovered with the first, most nonpolar fraction. Female extracts and fractions were analyzed by coupled gas chromatography–mass spectrometry. Cuticular hydrocarbon alkanes were identified as the extract components whose concentrations correlated best with male responses. Evidence of the pheromone's long persistence, low volatility, low polarity, and presence over the insect's entire body surface further supported the hypothesis that the pheromone was composed of one or more cuticular hydrocarbons.

Key Words—parasitoid, sex pheromone, *Roptrocerus xylophagorum*, Pteromalidae, bark beetle, Scolytidae, cuticular hydrocarbons, semiochemicals.

* E-mail: briansullivan@fs.fed.us

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INTRODUCTION

Sex pheromones are apparently widespread in the parasitic Hymenoptera and have been shown to occur in at least 16 families (Eller et al., 1984; Swedenborg and Jones, 1992a; Cormier et al., 1998). These pheromones mediate mate-finding and mating behaviors, including long-range orientation of males to females (Swedenborg and Jones, 1992a; Fauvergue et al., 1999; Jewett and Carpenter, 1999), concentration of male searching activity on substrates contacted by females (Kamano et al., 1989; Kainoh et al., 1991; Pompanon et al., 1997; Fauvergue et al., 1998), and short-range mate recognition and stimulation of male courtship behaviors (Vinson, 1978; Yoshida, 1978; Simser and Coppel, 1980; Takahashi and Sugai, 1982). Interest in parasitoid sex pheromones has been fueled in part by the practical needs of integrated pest management and the benefits offered by pheromone-baited traps as an efficient means for sampling and monitoring parasitoid populations in the field (Lewis et al., 1971; Powell, 1986; Reed et al., 1994; Jewett and Carpenter, 2001).

Roptrocerus xylophagorum (Ratzeburg) is an external parasitoid of the larvae and pupae of at least 12 economically important species of bark beetles (Coleoptera: Scolytidae) in North America, Australia, and Europe (Bushing, 1965; Mills and Schlup, 1989; Sullivan et al., 2000). My observations of male sexual behavior suggested the presence of a sex pheromone in this species; however, males did not demonstrate anemotaxis or other long-range orientation to live females in a Y-tube olfactometer. The following studies were devised to test the hypothesis that *R. xylophagorum* uses a close-range sex pheromone and to identify some of this pheromone's functional and chemical properties.

METHODS AND MATERIALS

Insects. Parasitoids were obtained from a stock culture initiated with insects from Alabama, Georgia, and Louisiana. Voucher specimens from this culture have been deposited at the University of Georgia Museum of Natural History. Parasitoids were collected daily from the window-illuminated side of a screen cage as they emerged from bolts of loblolly pine, *Pinus taeda* L., infested with the bark beetle, *Ips grandicollis* (Eichhoff) (Sullivan et al., 1999). Following collection, males and females were housed together in 250-ml Erlenmeyer flasks containing two to three light-duty paper wipers and stored in an incubator at 8°C, 40–90% relative humidity, and a 14L:10D photoperiod. Flasks were provisioned with honey and distilled water and removed from the incubator for approximately 1 hr daily to allow parasitoids to feed, take water, and mate. Male courtship behavior toward live females was observed inside the flasks or small glass enclosures and recorded.

Unless otherwise noted, parasitoid males and females utilized in bioassays had been collected from emergence cages three to five days previously. Ninety

percent of these females were found to have live spermatazoa in their spermathecae ($N = 30$). One day prior to use in bioassays, males were isolated from females into separate Erlenmeyer flasks held as above but at $25 \pm 2^{\circ}$C.

Bioassays. Male responses to surrogates of live females were observed in an olfactometer consisting of a glass tube (15 mm $\times$ 5 mm ID) sealed on one end with a disk of Teflon screening (0.1-mm mesh). Each female surrogate was mounted singly on an insect pin inserted lengthwise through a cork such that the surrogate was 6–9 mm from the cork's tapered end. Surrogates were either glass female "decoys" or the cadavers of freeze-killed female parasitoids. Glass decoys consisted of borosilicate glass bulbs (3–4 mm long $\times$ 1.5 mm diam.) constructed by melting the ends of Drummond 5-μl micropipets in a Bunsen burner (Figure 1). For cadaver surrogates, females were killed in a deep freezer and mounted lengthwise on insect pins by gluing their legs to the pin's shaft with a small amount of Elmer's Glue-All. After the glue had dried, extracts were applied to surrogates as a 10-μl aliquot delivered from either a micropipet or a Hamilton syringe. The extract was delivered as evenly as possible across the surface of each female surrogate. Normally, glass decoys were assayed two days following application of test extracts; cadavers were tested one day following mounting and application of test extracts to permit extraction solvents to evaporate completely. Unless noted otherwise, individual surrogate preparations were tested in four separate trials (each with a different male) and then replaced. No significant reduction in male responses was observed between the first and fourth trials with the same surrogate ($P = 0.136$, signed-rank test, $N = 201$).

At the beginning of each trial, a male parasitoid was gently aspirated into the glass tube using a length of flexible plastic tube ($\sim$30 cm long $\times$ 6 mm ID) sealed to the screened end of the glass tube. A mounted female surrogate was then presented to the male by inserting it into the open end of the glass tube. The surrogate's mounting cork was pushed firmly into the tube's mouth, thus stabilizing the surrogate within the glass tube and preventing male escape. The behavior of the enclosed male was then observed with a dissecting microscope under fluorescent laboratory lighting for 2.0 min.

For each trial, a response score was recorded as the sum of single points awarded for each of three observed behaviors: (1) climbing onto the surrogate while antennating its surface ("mounting"), (2) rapid vibration of the wings either while adjacent to or mounted on top of the surrogate ("fanning"), and (3) repeated thrusting of the exserted genitalia against the surrogate ("attempted copulation"). Males were tested once and then discarded. Individual experiments consisted of sequential assays of a related set of treatments grouped to allow statistical comparisons. Within each experiment, treatments were assayed in random order and with equal replicates of each treatment executed at any single testing session. Experiments were conducted between hours 4 and 11 of photophase at ambient laboratory conditions: $25 \pm 2^{\circ}$C and 30–60% relative humidity. Separate olfactometer tubes

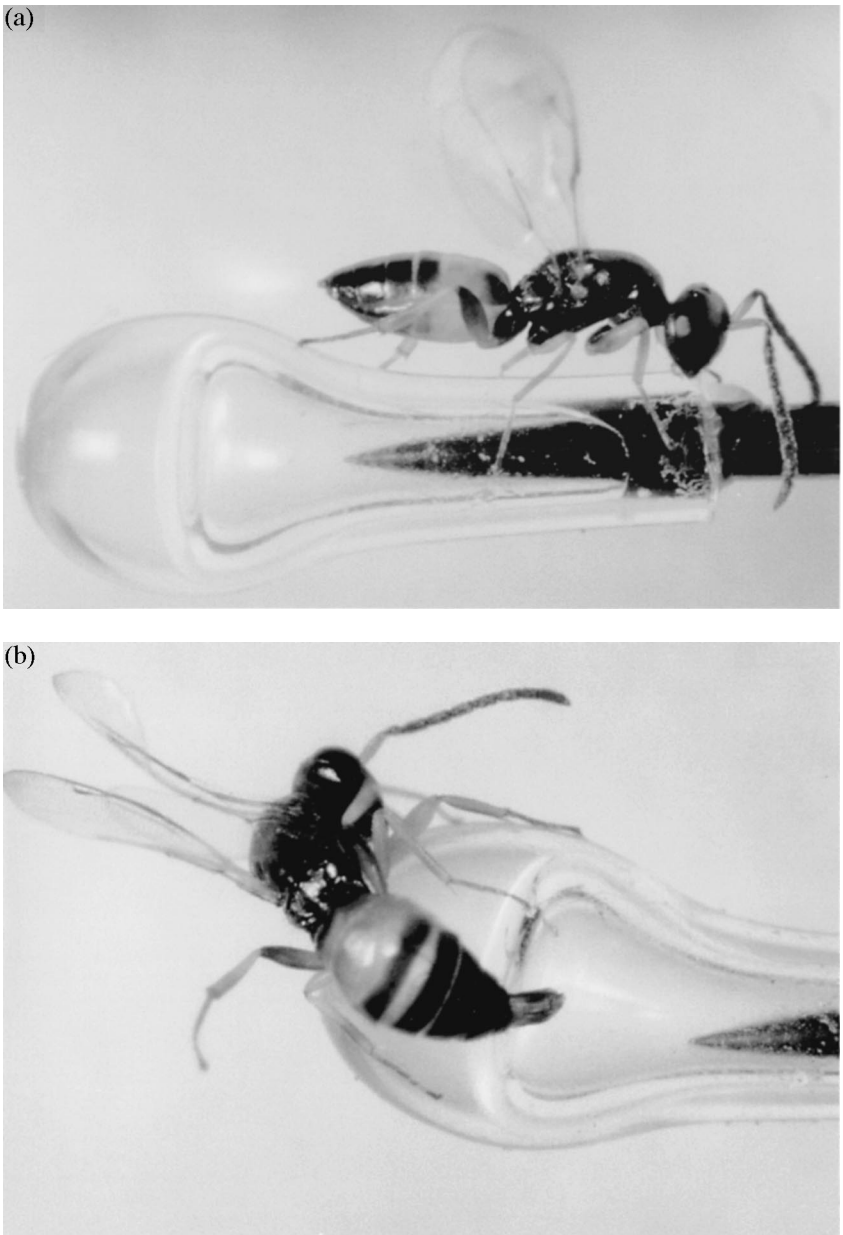


FIG. 1. Male *R. xylophagorum* exhibiting sexual behaviors in response to a glass bulb treated with a whole-body acetone extract of *R. xylophagorum* females: wing fanning (a) and copulation attempt (b).

were assigned to the different surrogate treatments, and olfactometer tubes were sonicated in a 1:1 acetone–ethanol mixture and oven dried for >2 hr at 120°C between test sessions. Experiments and treatments are described below.

Pheromone Stimulation of Male Sexual Behaviors. In the olfactometer, males ($N = 20$) were presented with female cadavers that were either (1) not manipulated, (2) extracted to remove all traces of pheromone, or (3) extracted and then treated with one female equivalent (1 FE) of female extract. Female extract was obtained by steeping 10 freezer-killed females in 50 μl acetone for 15 min, then rinsing the insects with another 50 μl acetone and combining the rinse with the original extract (1 FE = 10 μl extract). Cadavers used in treatments 2 and 3 above were further extracted: twice in 10 ml hexane, acetone, and 95% ethanol for >1 hr each with gentle agitation on a shaker table.

Pheromone Characteristics. In separate experiments, males were presented with: (1) glass decoys treated with either a hexane, acetone, or methanol extract (1 FE) of whole parasitoids, (2) decoys treated with sequential dilutions of a 1 FE acetone extract of whole females (1.0, 0.1, 0.01, 0.001, or 0.0001 FE), or (3) decoys that had been treated with a 0.01 FE acetone extract and then stored in a dark cabinet in the laboratory for 2–6 hr, or one, two, four, or seven days ($N = 20$, 20, and 32, respectively).

Source of Pheromone. Isolated body regions were extracted sequentially to distinguish between pheromone that may have spread across the entire body surface from its site of origin and that present in exocrine organs within or beneath the cuticle. The abdomens of 10 freezer-killed females were separated from heads/thoraces with a scalpel, and the two body regions were placed into separate vials and extracted three times in acetone: (1) 5 min with 100 μl, then (2) 15 min with 100 μl, and finally (3) thoroughly macerated with a glass rod and extracted with 1.0 ml for 15 min. Prior to application to decoys, extracts 1 and 2 were diluted 1/10 in acetone (0.1 FE = 10 μl). Extract 3 was spun at 2000 rpm in a clinical centrifuge to remove particulates and then used undiluted. In the olfactometer, males ($N = 20$–28) were presented with glass decoys treated with one of the three extracts of either body region. Each extraction was tested as a separate two-treatment experiment.

Effect of Female Mating and Age on Pheromone Production. Parasitoid pupae were dissected from *I. grandicollis*-infested pine bolts from the stock culture, placed into gelatin capsules, incubated in the dark at 29°C and 70–80% relative humidity, and inspected daily. On the day of eclosion, females were either extracted immediately or transferred in groups of up to eight individuals into housing flasks as described previously. Half of these flasks contained 3-5-day-old males ($N = 12$–15) collected from the emergence cage; the remaining flasks did not contain males. Flasks were maintained at 25 ± 2°C, 40–80% relative humidity, and a 14L:10D photoperiod for three to five days, and then the females were removed and extracted. To estimate the proportion of mated females in the mixed-sex flasks at the time of extraction, 18 females were dissected live and the spermathecae examined.

Individual parasitoids were extracted whole in acetone (2×100 μl) for 15 min (0.05 FE = 10 μl). Males ($N = 32$) were presented with glass decoys treated with the extract of: (1) a 1-day-old virgin male, (2) a 1-day-old virgin female, (3) a 3- to 5-day-old virgin female, (4) a 3- to 5-day-old female from a mixed-sex flask, or (5) a female that had naturally emerged three to five days earlier from a pine bolt in the stock culture. Eight decoys each bearing the extract of an individual insect were assayed per treatment (four males tested per decoy).

In a separate experiment, males ($N = 32$) were presented with cadavers of females that had emerged from culture bolts either 3–5 or 14–16 days earlier. Females in the 14 to 16 day category were removed from the 8°C incubator at five days and then maintained in culture flasks at 25 ± 2°C, 40–80% relative humidity, and a 14L:10D photoperiod for an additional 9–11 days. Females in the three to five-day category were kept consistently at 8°C prior to freezing and mounting.

Effect of Recent Female Contact on Male Responsiveness to Pheromone. Males were isolated from females for varying durations prior to being assayed for their response to decoys treated with 0.1 FE acetone extract of whole females. Male test subjects were held in housing flasks with ~50 females before being isolated into clean flasks. Males ($N = 30$) experiencing four different separation intervals were assayed: 0 hr (removed from mixed-sex flask immediately before each trial), 1–2 hr, 5–9 hr, and 18–36 hr. The same decoy was used in up to eight trials.

Activity of Fractions of Female Extract. A hexane extract of whole females was fractionated using low-pressure liquid chromatography, and the resulting fractions were bioassayed. A Pasteur pipet was plugged at its tip with silanized glass wool and then wet-packed with silica gel (1.0 g, 70–230 mesh, Merck grade 7754, conditioned >1 hr at 130°C) in 40% ethyl ether in hexane. The packed column was rinsed with 10 ml hexane prior to loading with sample (50 μl of a 100-μl hexane extract of 10 females, i.e., 5 FE). Fractions were eluted sequentially with 5 ml of hexane; 5% ether in hexane; 30% ether in hexane; ether; acetone; and methanol. Prior to application to decoys, each fraction was evaporated under nitrogen to a volume of 0.5 ml, and a portion of the original extract was diluted 1/10 in acetone (so that 10 μl = 0.1 FE for all extracts and fractions). Males ($N = 16$) were presented with glass decoys treated with each of the six fractions singly, the six fractions in combination (10 μl of each), or the original, unaltered extract.

Female Deposition of Pheromone on Surfaces. Ten females were confined for 0.5 hr inside a 4-cm-diam. plastic Petri dish bottom covered with a 15-cm-diam. glass plate. During this time, females walked across and/or stood motionless on the glass surface. Control glass plates were prepared similarly but with no females present. Two concentric circles were drawn on the reverse side of each plate: the inner circle marked the position of the dish rim and the outer was 7 cm diam. The Petri dish and females were removed, and the glass plate was suspended vertically against a white surface (dish side out). Within 1 hr of female removal, individual

male parasitoids were released onto the plate surface (inside the bottom third of the inner circle) and observed for 5 min. The total time each male spent in motion within the outer circle was measured with a stopwatch. Four female-exposed and blank plates were assayed with 6–10 males each.

Chemical Analyses. Portions (100 μl each) of the extracts and fractions utilized as decoy baits were concentrated under nitrogen to approximately 2–5 μl, and 1 μl was then injected splitless into a Hewlett-Packard 6890 gas chromatograph coupled to a model 5973 mass-selective detector (GC-MS). The column was a Hewlett-Packard HP-1 (polydimethylsiloxane; 50 m $\times$ 0.2 mm ID, 0.11-μm film thickness); the temperature program was 60°C for 1 min, then 10°C/min to 200°C, then 6°C/min to 310°C, then isothermal for 20 min. Each sample was spiked with two internal standards (11 ng 1,3,5-triphenyl benzene and 9 ng α-humulene) prior to concentration. Components of the extracts–fractions were identified by their mass spectra and retention time matches to known standards when available. Each component was quantified by dividing the integration area for one of its diagnostic ions by the integration area of a diagnostic ion of one of the internal standards. The following decoy baits were analyzed: acetone extract of 1-day-old virgin males, acetone extracts of 1-day- and 3- to 5-day-old virgin females, hexane extract of naturally emerged, presumably mated females, and all six silica gel fractions of the hexane extract.

Statistical Analyses. Response scores for the two to four males tested with each surrogate were averaged, and these means were utilized as individual data points in statistical analyses. Significant differences among treatment means were identified with the nonparametric Kruskal-Wallis ANOVA and Mann-Whitney rank sum tests. The Student-Newman-Keuls test was employed for all-pairwise comparisons among treatments ($\alpha = 0.05$; SigmaStat, 1997). Associations between the concentrations of bait constituents and male responses to those baits (i.e., the mean response score for that bait) were assessed with Spearman's rank order correlation (SigmaStat, 1997).

RESULTS

Male Sexual Behavior. Courtship behavior by males toward live females consisted of a characteristic succession of steps. A male would initiate courtship either after chance antennal contact with a female (during what appeared to be nondirected, random movements) or after coming within $\sim$2 cm of a female. The male would approach with accelerated, sinuous movements while fanning his wings almost continuously. He would then mount the female, typically from the side, while making constant antennal contact. Once on top, the male aligned his body above the female's with his head directly above hers. Sometimes the male would pivot his body several times before settling in the typical alignment. The

aligned male then initiated a predictable sequence of behaviors: (1) a brief burst of wing fanning, (2) a nodding motion of the head in which the mouthparts were extruded and stroked forward and upward above the female's antennae, and (3) a motionless pause approximately equal in duration to behaviors 1 and 2. Step 2 was often accompanied by sweeping movements of the male's antennae slightly towards one another and upward; occasionally the male's antennae would contact and noticeably move the female's antennae. This complete sequence was typically repeated two to five times and immediately followed either by male attempts at copulation or abandonment of the female. If copulation attempts failed, often the male would reposition himself above the female and recommence the sequence.

Bioassays. In the glass-tube olfactometer, males responded to female cadavers with wing fanning, mounting, and copulation attempts. Cadavers extracted with a series of organic solvents elicited relatively few male responses, but their activity was largely restored after treatment with 1 FE acetone extract (Figure 2). Males responded to extracted cadavers with mounting and antennation only; only the nonextracted and extract-treated cadavers elicited wing fanning and copulation attempts.

Glass bulb decoys treated with 1 FE extract likewise stimulated wing fanning, mounting, and copulation attempts (Figure 1). However, methanol extracts of females elicited significantly lower levels of response than either hexane or acetone extracts (Figure 3). Acetone extracts of females induced male responses in a dose-dependent manner within the concentration range of 0.0001 FE to 1 FE (Figure 4).

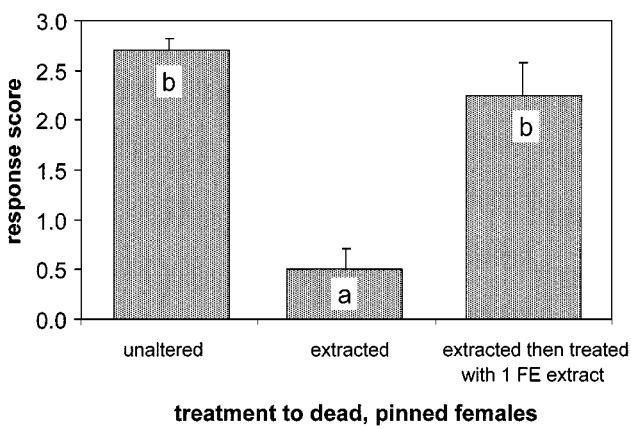


FIG. 2. Mean response scores (+SE) of individual male *R. xylophagorum* ($N = 20$) reacting to pin-mounted female cadavers that were either not manipulated, extracted repeatedly in organic solvents, or extracted and then treated with whole-body extract (1 FE) of a female. Means with the same letter are not significantly different (Student-Newman-Keuls test; $\alpha = 0.05$).

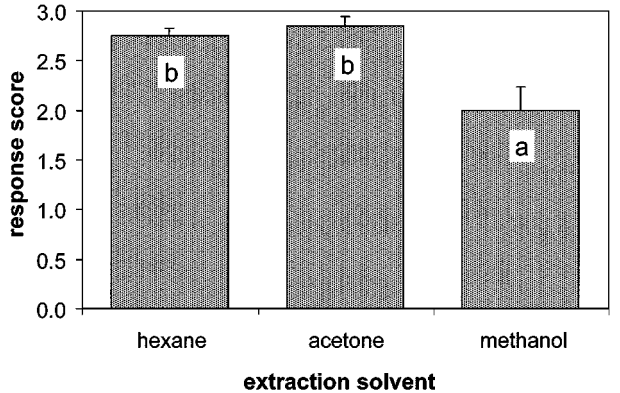


FIG. 3. Mean response scores (+SE) of individual male *R. xylophagorum* ($N = 20$) reacting to glass bulb decoys treated with either a hexane, acetone, or methanol extract (1 FE) of a female. Means with the same letter are not significantly different (Student-Newman-Keuls test; $\alpha = 0.05$).

Glass decoys treated with 0.01 FE acetone extract gradually lost activity over a seven-day period (Figure 5).

Male responses to decoys treated with the initial extracts of either abdomens or heads/thoraces of females were essentially identical, while decoys treated with the second consecutive extract of heads/thoraces had a marginally higher level of activity than the second consecutive extract of abdomens ($P = 0.053$; Figure 6).

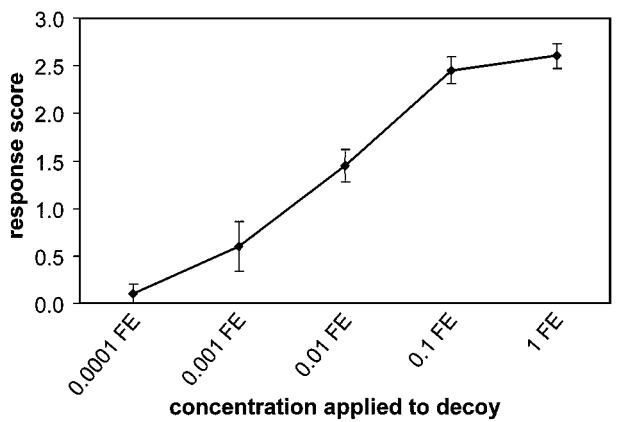


FIG. 4. Mean response scores (±SE) of individual male *R. xylophagorum* ($N = 20$) reacting to glass bulb decoys treated with the serial dilutions of an acetone extract of females (0.0001–1.0 FE).

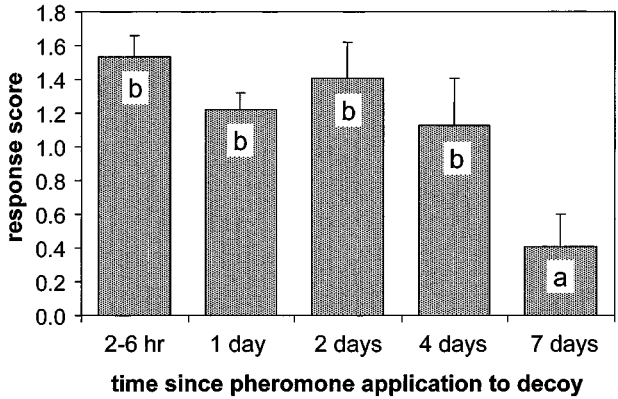


FIG. 5. Mean response scores (+SE) of individual male *R. xylophagorum* ($N = 32$) reacting to glass bulb decoys treated with female extract (0.01 FE) at different time intervals prior to testing. Means with the same letter are not significantly different (Student-Newman-Keuls test; $\alpha = 0.05$).

This difference in the activity of the second extracts was most visible in the number of males making copulation attempts: 12 for head/thorax-treated decoys vs. 1 for abdomen-treated decoys. Maceration and further extraction of the two body regions yielded extracts with relatively low levels of activity that did not differ significantly.

Live spermatozoa were found in the spermathecae of 12 of 18 females that had been housed with males for three to five days following eclosion in gelatin capsules. Extracts of these females, of 3- to 5-day-old virgin females from single-sex flasks, and of females naturally emerged from infested bolts elicited roughly similar

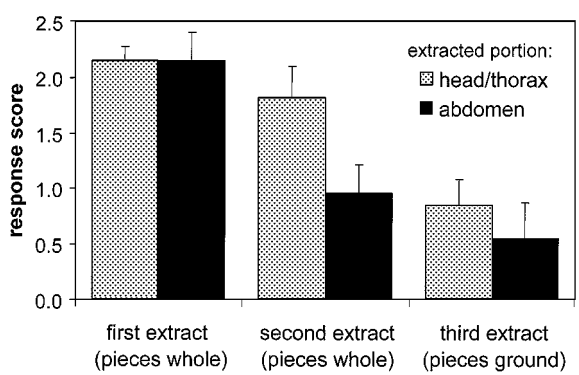


FIG. 6. Mean response scores (+SE) of individual male *R. xylophagorum* ($N = 20-28$) reacting to glass bulb decoys treated with one of three sequential extractions of either the head/thorax or abdomen of females (0.1 FE).

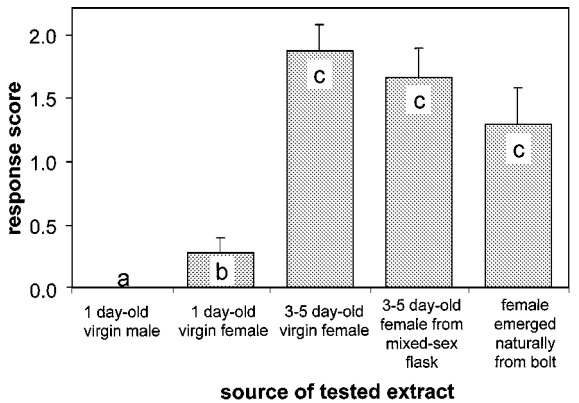


FIG. 7. Mean response scores (+SE) of individual male *R. xylophagorum* ($N = 32$) reacting to glass bulb decoys treated with the acetone extracts (0.05 FE) of 1-day-old virgin males, 1-day-old virgin females, 3- to 5-day-old virgin females, 3- to 5-day-old females that had been housed in mixed sex flasks, or females that had emerged naturally from host-infested pine bolts and were presumably mated. Insects in all treatments except the last had been isolated as pupae from host-infested bolts. Means denoted with the same letter are not significantly different (Student-Newman-Keuls test; $\alpha = 0.05$).

levels of male responses; simultaneously, these three treatments had significantly greater activity than extracts of either 1-day-old virgin males or females (Figure 7). Extracts of 1-day-old virgin females showed significantly greater activity than extracts from 1-day-old virgin males. Male response scores elicited by cadavers of females that had emerged from bolts either three to five days earlier (2.50 ± 0.23) or 14–16 days earlier (2.53 ± 0.15) did not differ significantly.

Mean response to extract-treated decoys was significantly lower for males removed from mixed-sex flasks immediately before bioassay than for males isolated from females 1–2, 5–9, or 18–36 hr prior to bioassay (Figure 8). Males isolated 1–2 hr prior to bioassay were intermediate in responsiveness between males with either longer or shorter periods of isolation.

Most of the activity present in female extract was recovered with the first hexane fraction on silica gel (Figure 9). Male responses did not differ significantly between decoys treated either with all six fractions or with the first fraction alone, indicating the absence of synergism between any of the fractions.

Males remained in motion within a 7-cm-diam. zone on a glass plate for significantly longer durations when females had previously been confined within the zone than when they had not (Figure 10; $P < 0.001$, Mann-Whitney rank-sum test). Half the males placed onto female-exposed plates exhibited wing-fanning behavior, and many such males exhibited accelerated, sinuous movements in and around female-contacted surfaces.

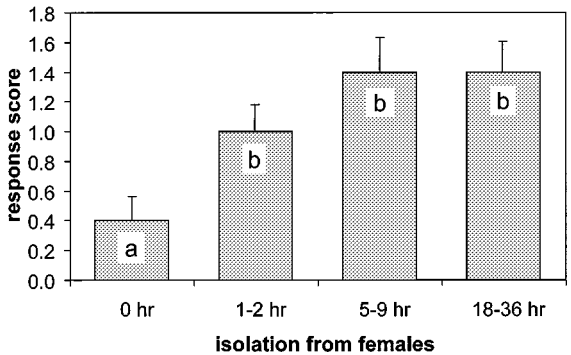


FIG. 8. Mean response scores (+SE) of individual male *R. xylophagorum* ($N = 32$) that had been removed from mixed-sex flasks at variable intervals prior to testing with glass decoys treated with female extract (0.1 FE). Means denoted with the same letter are not significantly different (Student-Newman-Keuls test; $\alpha = 0.05$).

Chemical Analyses. The 68 largest GC peaks in female extract (i.e., all peaks >0.2% the integration area of the largest peak) were quantified for the examined decoy baits, and identifications were made for 28 peaks exhibiting a strong degree of association (Spearman rank-order correlation coefficient >0.75) between their concentrations and male response scores to the individual baits (Table 1). Structures

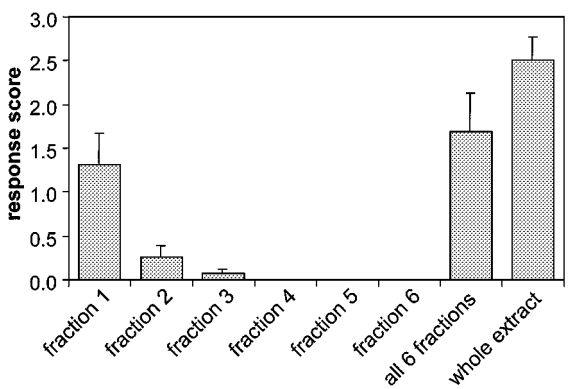


FIG. 9. Mean response scores (+SE) of individual male *R. xylophagorum* ($N = 16$) reacting to glass bulb decoys treated with a hexane extract of females, each of six LC fractions of the extract, or all six fractions simultaneously. Extract fractions were eluted sequentially with hexane (fraction 1), 5% ether in hexane (fraction 2), 30% ether in hexane (fraction 3), ether (fraction 4), acetone (fraction 5), and methanol (fraction 6). Extract and fractions were applied at 0.1 FE per decoy.

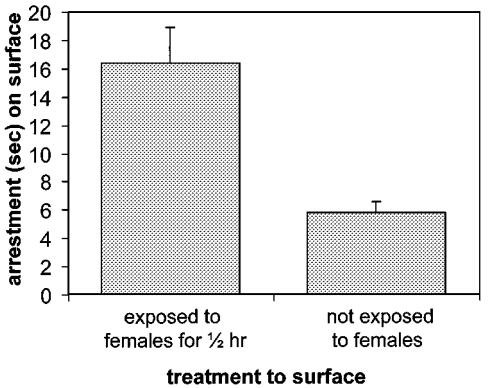


FIG. 10. Mean time (+SE) spent by male *R. xylophagorum* ($N = 29-40$) in motion within a 7-cm-diam. zone of a glass surface that had or had not been contacted by walking females. Male arrestment time differed significantly between the two treatments (Mann-Whitney test; $P < 0.001$).

were determined for all 28 of the correlating peaks based on retention times and interpretation of mass spectra (Blomquist et al., 1987; Carlson et al., 1998). These compounds consisted entirely of alkanes with a 27- to 33-carbon backbone and no, one, or two methyl branches.

DISCUSSION

Female *R. xylophagorum* apparently possess a sex pheromone capable of stimulating courtship behavior and mating in males. Removal of chemical cues from female cadavers by solvent extraction largely eliminated sexual behavior directed toward them, while restoration of chemical cues through the reapplication of female extracts restored male responses. Male response to the pheromone was minimally altered by the absence of visual and tactile cues normally associated with a female. Males responded with the complete courtship sequence (including body alignment and head nods) to pheromone-treated glass decoys, although these decoys differed substantially in color, shape, and texture from female parasitoids. However, the courtship sequence was often terminated at various stages prior to attempted copulation, and this likely resulted from the absence of receptivity signals or other cues from the female surrogate (Van Den Assem, 1986). Pheromone-based stimulation of male courtship behaviors (including wing fanning and copulation attempts) in the absence of normal visual/tactile cues associated with females has been reported in other parasitic Hymenoptera (Obara and Kitano, 1974; Kitano, 1975; Robacker et al., 1976; Vinson, 1978; Yoshida, 1978; Takahashi and Sugai, 1982; Shu and Jones, 1993).

TABLE 1. CONSTITUENTS OF FEMALE EXTRACT WITH STRONG ASSOCIATION BETWEEN THEIR CONCENTRATIONS AND RESPONSE SCORES OF MALE *R. xylophagorum* (SPEARMAN'S CORRELATION COEFFICIENT >0.75)

Kovats index	Compound name	Correlation coefficient[a]	% of cuticular hydrocarbons[b]	Gender association[c]
2700	*n*-Heptacosane	0.957	3.5	F > M
2743	7-Methylheptacosane	0.861	0.6	—
2752	5-Methylheptacosane	0.829	1.3	F
2774	3-Methylheptacosane	0.787	1.6	F
2800	*n*-Octacosane	0.875	0.5	F
2811	3, 7-Dimethylheptacosane	0.814	1.0	F
2833	11-, 12-, 13-, and 14-Methyloctacosane	0.771	0.3	—
2846	6-Methyloctacosane	0.814	0.2	—
2859	4-Methyloctacosane	0.874	0.4	—
2893	4,16-Dimethyloctacosane	0.954	0.3	—
2900	*n*-Nonacosane	0.875	6.0	F > M
2911	3, 7-Dimethyloctacosane	0.902	0.5	—
2930	11-, 13-, and 15-Methylnonacosane	0.906	10.0	F > M
2942	7-Methylnonacosane	0.814	2.8	—
2952	5-Methylnonacosane	0.814	1.9	F
2961	11,15-Dimethylnonacosane	0.902	2.1	—
2967	9,15-Dimethylnonacosane	0.803	1.8	—
2976	3-Methylnonacosane	0.814	11.0	F > M
3015	3, 7-Dimethylnonacosane	0.814	21.0	F > M
3031	13-, 14-, and 15-Methyltriacontane	0.944	0.3	—
3060	2-Methyltriacontane	0.874	0.6	—
3132	13- and 15-Methylhentriacontane	0.850	5.1	F&M
3158	13,17-Dimethylhentriacontane	0.814	7.2	F
3182	5,17-Dimethylhentriacontane	0.902	2.1	—
3209	3,9-Dimethylhentriacontane	0.902	3.7	—
3329	17-Methyltritriacontane	0.874	0.2	—
3355	15,19-Dimethyltritriacontane	0.902	1.2	—
3381	5,17-Dimethyltritriacontane	0.787	1.1	—

[a] Spearman's rank-order correlation coefficient between average male response score and quantity of compound identified in tested bait. Baits subjected to analyses included extracts of 1-day-old virgin males, 1- and 3- to 5-day-old virgin females, mated females, and six silica gel fractions of this latter extract.

[b] Percentage of total cuticular hydrocarbon composition identified in female *R. xylophagorum*. Only those hydrocarbons with correlation coefficient >0.75 are shown (88.3% of total).

[c] Gender association of individual compounds identified in the cuticular hydrocarbons of North American populations of *R. xylophagorum* (Espelie et al., 1996). Treatment abbreviations are F = only in females, F > M = female concentrations greater than four times those in males, F&M = present in roughly similar concentrations in both sexes, — = not reported in Espelie et al. (1996).

The courtship-stimulating pheromone may also mediate mate-finding in *R. xylophagorum*. Males coming into contact with residue deposited on a glass surface by walking females confined their movements to its vicinity for up to 45 sec while apparently searching for females. The residue likewise induced male wing fanning, a behavior, which, in addition to triggering receptivity in females (Kitano, 1975; Van Den Assem, 1986), may also enhance orientation toward sources of female odors (Vinson, 1972, 1978). Males of other parasitoid species exhibit similar, mate-finding-related behaviors (e.g., arrestment, accelerated movement, increased turning rate) on substrates previously contacted by females (Kamano et al., 1989; Pompanon et al., 1997; Fauvergue et al., 1998). Substrate-borne pheromone residues may concentrate searching by males in areas frequented by females, increasing the probability of sexual encounters. Although lacking the ability to attract males over long distances, substrate-borne sex pheromones can be adequate for ensuring a high rate of insemination for female parasitoids (Fauvergue et al., 1995).

The gradual recovery in male responsiveness to sex pheromone following removal from mixed-sex flasks indicated that male courtship behaviors can be temporarily inhibited by continuous contact with females or their environment. The inhibition in male responsiveness was possibly caused by habituation to the female's sex pheromone (Robacker et al., 1976; Askari, 1979) or may have been a reaction to unsuccessful courtship attempts with unreceptive females (Van Den Assem, 1986).

Sex pheromones in hymenopterous parasitoids originate in the abdomen (Tagawa, 1977; Vinson, 1978; Yoshida, 1978; Askari and Alishah, 1979), the head and/or thorax (Takahashi and Sugai, 1982; Kainoh and Oishi, 1993), or both regions (Vinson, 1972), and pheromone-producing glands have been identified in both the abdomen and head (Tagawa, 1977; Weseloh, 1980; Syvertsen et al., 1995; Jones, 1996). Parasitoid pheromones may have their origin in a localized gland but spread across the insect's entire body either passively or as a result of grooming (Tagawa, 1977). The results presented here suggest that the sex pheromone of *R. xylophagorum* was uniformly present across the body surface of females, but do not clearly identify a single body region as the site of pheromone production.

Mating by female parasitoids can cause an abrupt termination of male responses through an apparently copulation-induced change in the release rate or composition of the female pheromone (Fauvergue et al., 1995; McNeil and Brodeur, 1995; Pompanon et al., 1997; Schwörer et al., 1999). *Roptrocerus xylophagorum* males did not respond differently to extracts from virgin females than to extracts from females that had been housed with males, suggesting that mating does not alter pheromone presence on females. However, dissections of spermathecae indicated that some of the females from mixed-sex flasks had probably never mated, and their unintentional inclusion in the tests may have partially obscured changes in male responses.

Concentrations of pheromone on female *R. xylophagorum* apparently did not diminish with age, since males responded equally to cadavers of females that were either younger (3–5 days old) or older (14–16 days old) than the average life span of six to eight days (Samson, 1984; Matthews and Stephen, 1997). The life-long presence of active sex pheromone on female *R. xylophagorum* is not a unique phenomenon within the parasitic Hymenoptera (Vinson, 1978; Swedenborg and Jones, 1992b). There is little evidence to suggest that persistence of sexual attractiveness enhances the fitness of female *R. xylophagorum.* This species is reported to mate immediately after emergence and only once (Bushing, 1967), and the extreme infrequency with which I have observed apparently receptive *R. xylophagorum* females seems to corroborate this report. The persistence of pheromone on female *R. xylophagorum* might be an incidental consequence of the low volatility and high stability of semiochemicals produced earlier in life, and little or no active synthesis or release of pheromone may be necessary for the pheromone's activity to persist throughout the female's life span.

The only extract constituents possessing a strong quantitative association with male responsiveness were apparently cuticular hydrocarbons. All were long-chain alkanes removed from whole insects with relatively brief steeping in hexane and had been previously identified in the cuticular hydrocarbons of other insects (Blomquist et al., 1987; Carlson et al., 1998). Espelie et al. (1996) reported numerous qualitative and quantitative differences in the cuticular hydrocarbon composition of male and female *R. xylophagorum* and suggested that these differences might play a role in close-range mate recognition. Several of the compounds correlating quantitatively with male responsiveness in my study were previously identified by Espelie et al. (1996) as being associated primarily or uniquely with female *R. xylophagorum* (Table 1).

The bioassay results support the hypothesis that the sex pheromone is composed of cuticular hydrocarbons, which function as close-range sex pheromones in several orders of insects (Howard, 1993). As expected for cuticular hydrocarbons, the pheromone was apparently distributed over the entire body surface. It was highly nonpolar, eluting mainly with the first, hexane fraction on silica gel, and it exhibited significantly lower solubility in methanol relative to hexane and acetone. Likewise, the long persistence of the pheromone on objects was consistent with the typical stability and extremely low vapor pressure of long-chain, saturated hydrocarbons. Bioassays with synthetic hydrocarbons will be necessary to confirm the role of these compounds in mediating sexual behavior in *R. xylophagorum*.

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EVIDENCE FOR A COMPOUND IN COMSTOCK-KELLOG GLANDS MODULATING PREMATING BEHAVIOR IN MALE DESERT LOCUST, *Schistocerca gregaria*

PETER G. N. NJAGI[1,*] and B. TORTO[1,†]

[1]*The International Centre of Insect Physiology and Ecology (ICIPE)*
P.O. Box 30772, Nyayo Stadium
00506, Nairobi, Kenya

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Abstract—The Comstock-Kellog glands in adult females of certain acridid species, including the desert locust, *Schistocerca gregaria* have been implicated as a source of volatiles that play a role in mating behavior. A dichloromethane extract of the glands was analyzed for metabolites by gas chromatography, coupled gas chromatography–electroantennographic detection, and mass spectrometry. Coupled gas chromatography–electroantennographic detector (GC-EAD) analysis revealed a component that elicited an electroantennogram response from the antenna of adult male *S. gregaria*. The compound was identified by GC-MS as pentanoic acid. The levels of the compound in the gland extract varied with age of female locust; it was present in detectable amounts only in 14- to 16-day-old females. In bioassays, pentanoic acid significantly stimulated pre-mating behavior in male desert locust. These results are discussed in relation to the biology of the locust.

Key Words—Pheromone, mating behavior, *Schistocerca gregaria*, Acrididae, Comstock-Kellog gland, pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid, decanoic acid.

INTRODUCTION

Acridids in the subfamily *Cyrtacanthacridinae* exhibit precopulatory behavior as part of their mating repertoire (Uvarov, 1966). These behaviors may involve various postures, nonlocomotory movements, and gestures. In the desert locust, *Schistocerca gregaria*, the behavior is generally characterized by a slow approach

* To whom correspondence should be addressed. E-mail: pnjagi@icipe.org
† Present address: USDA/ARS/CMAVE, 1700 SW 23rd Drive, Gainesville, Florida 32608.

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of the male to the female, frequently interrupted by "freezing" in a semistilted posture, and rhythmic movements of the maxillary palps (Uvarov, 1977), antennation, antenna- and eye-cleaning, scanning, and body vibration (Inayatullah et al., 1994). Other male sexual behaviors may involve production of "short" and "long" bursts of sound by stridulation of both pairs of wings (Loher, 1959) or rapid vibration of the wings under the tegmina, described by some authors as stationary wing-fluttering (Uvarov, 1977).

In female locust of some species in the subfamily, precopulatory behavior may involve extrusion of the Comstock-Kellog glands (CKGs). These are a pair of membranous eversible organs with folded walls and glandular epithelium that are located at the anterior of the floor of the genital pouch (Thomas, 1965; Uvarov, 1966; Szopa, 1981). Over the years, there has been debate as to whether the glands function in mate attraction or in oviposition (Whitman, 1990). Nevertheless, experiments have been conducted on only a few species. The first experiment was conducted by Eisner et al. (1966), who showed that in the grasshopper *Romalea microptera*, secretions from the glands cause tanning of eggs and froth of the egg pod. In later experiments, excision of the glands from mature virgin females of another grasshopper, *Melanopus sanguinipes*, did not affect their coupling with males (Pickford and Gillott, 1972). Similarly, removal of the CKGs from females of *S. gregaria* did not affect their oviposition behavior and egg pod formation (Szopa, 1981). In these grasshopper species and the desert locust, results from experiments do not preclude the possibility that the glands may play a role in other aspects of the mating behavior. It has been suggested that in the desert locust the glands may release odors that attract males (Uvarov, 1966, 1977). Despite the speculated role of the CKGs, no chemical analysis has been carried out on secretions of the glands.

In the present study, we employed gas chromatography (GC), coupled GC-electroantennography, GC-mass spectrometry, and behavioral assays to determine (1) the chemical composition of the extract from the gland, (2) whether there is evidence of sensory detection of extract components, (3) whether there is evidence of behavioral response to physiologically active compounds, and (4) whether there are age-related variations in the amounts of the active components?

METHODS AND MATERIALS

Insects. Crowd-reared (gregarious) desert locusts, *S. gregaria*, were from the ICIPE colony that originated from a stock obtained from the Desert Locust Control Organization for Eastern Africa (DLCO-EA) in Addis Ababa, Ethiopia. The colony had been propagated in the insectary since 1991, and locusts were reared as described in Obeng-Ofori et al. (1993), Torto et al. (1994), and Njagi et al. (1996). Batches of 300–400 hundred locusts of both sexes (male to female ratio *ca.* 1:1) were bred in cages made of light aluminum sheets ($50 \times 50 \times 50$ cm) that had wire mesh ventilation ports (14×15 cm) on two sides and the top (Ochieng-Odero et al.,

1994). Locusts in each cage developed synchronously from one stage to the next and were all of the same age. Cages with locusts were kept in a temperature-controlled room maintained at 30–35°C and a light–dark cycle of 12:12 hr. The room was aerated by a duct system maintained at a negative pressure. Insects were fed *ad libitem* fresh wheat (*Triticum* sp.) seedlings and wheat bran, provided daily.

Gland Extracts. Female locusts, 1–3, 7–9, 14–16, and 20–22 days old after final molt and held in glass jars, were kept in a freezer at −15°C prior to dissection to remove the glands. The CKGs were located as illustrated by Szopa (1981), and for each age group, glands from 15 females were dissected out. The glands were extracted in 2 ml HPLC grade dichloromethane (99.9%; Aldrich Chemicals, Gillingham, UK) in a vial kept overnight (*ca.* 18 hr.) in a refrigerator at 4°C. The extracts were filtered through a clean glass wool plug in a Pasteur pipet, dried over anhydrous sodium sulfate, and stored in a freezer at −15°C until used. For each age group, five such extract samples were prepared.

Analysis of Extracts. Extracts were concentrated to 100 μl under a stream of nitrogen at 0°C. For GC analysis, 4 μl of each extract were injected onto the capillary column. In GC-EAD tests, we used 8 μl since the GC had a 1:1 ratio splitter to the EAD and the detector. The GC analyses were performed on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with a flame ionization detector and an HP capillary column (Carbowax 20 M, 50 m × 0.2 mm ID × 0.2 μm). Nitrogen was used as the carrier gas at a flow rate of 0.35 ml/min. In GC, GC-EAD, and GC-MS analyses, the column temperature was set at 60°C for 10 min, programmed at 5°C/min to 180°C and held for 5 min, then at 10°C/min to 220°C and held for 10 min. Chromatographic peaks for the GC analysis were integrated with an HP 3396 Series II integrator. GC-MS analysis was carried out on a VG Masslab 12-250 mass spectrometer (EI, 70 eV) coupled to an HP 5790 gas chromatograph. The identities of the components in the extracts were confirmed by GC-EAD and GC-MS analyses of authentic samples.

In GC-EAD analysis, preparation of the mature male locust antenna followed Torto et al. (1994). The FID and EAD signals were monitored synchronously by using a program on an EAD interface card (Syntech) installed in a PC (Harvard Professional Computer, American Megatrends Inc.). The FID signal was also plotted on an HP 3396 Series II integrator at 0.5 cm/sec.

Still Air Glass Jar Bioassay. The glass jar (6.5 cm ID and 19 cm high) had a perforated metal top covered with a wire mesh. A piece of wire mesh was placed inside at an inclined angle for climbing. The dispenser for the test material was a Pasteur pipet (11. 0 cm long × 0.5 cm ID at the wide end) with one end drawn to a short tapering tip (≅1.0 cm from the shoulder to the shank) with a tip diameter of 0.2 cm. A small ball of cotton wool plugged into the tip to a depth of 0.3 cm. Aliquots of 1.0 and 2.0 μl (equivalent to 0.5 and 1.0 μg of pentanoic acid, respectively) of a stock preparation of the acid (>99% pure; Aldrich Chemicals), in polyethylene glycol 200 (PEG) (Aldrich Chemicals)

(to slow down the evaporative release of the compound), were each pipetted onto the cotton wool plug. A sexually mature male or female locust (16–20 days old after final molt) or a sexually immature adult male (seven to nine days after final molt) was placed into the glass jar and left to settle at the bottom. After 2 min, a pipet with the test material was inserted through the wire mesh of the cover on top of the jar and pushed about 6.0 cm into it. At this point, the dispensing tip of the pipet was about 2.0 cm from the top of the slanting wire mesh strip. For the control, the cotton wool was impregnated with a similar volume of PEG, and in both cases, the behavior of the locust inside the jar was monitored for the subsequent 15–20 mins. The insects for control and treatment tests were drawn from the same batches of gregarious locusts reared as outlined above, and each was observed individually. Glass jars were thoroughly cleaned with a detergent and dried in an oven prior to reusing them to avoid contamination. Preliminary studies showed that in response to our treatment, locusts performed one of three types of behaviors:

1. An insect moved to within 1–2 cm from the tip of the pipet dispenser and stayed for a minimum of 5 min with the body halfway between the "stilted" and "crouching" postures (Uvarov, 1977). In the former posture, the locust raised its whole body above the wire mesh with the tibiae of the hind legs slightly folded such that the femora lay below and almost parallel to the top of the elytra. When crouching, the insect had its body pressed against the substrate with the hind femora and tibiae folded at an angle and pointing upwards. This was accompanied by vigorous mandibular and maxillary palp movements, preening of antennae with foretarsi, and, on rare occurrences, stationary wing-fluttering (see 3 below). Most of these activities are elements of the male precopulatory behavior (PCB) as described by Uvarov (1977) and are often elicited in sexually mature males prior to mounting females.

2. An insect moved to within 3–5 cm from the tip of the dispenser and stayed stationary in semistilted posture and/or almost crouching without noticeable movement of other body parts. This is interpreted as nonsexual behavior, possibly triggered by the presence of the dispenser in the field of vision of the locust and has been denoted as secondary arrestment (SAR) in this paper.

3. Stationary wing-fluttering (SWF) consisted of rapid movements of the elytra and the wings raised high above the abdomen as in flight while the insect perched on the wire mesh or on the wall of the jar. A bout of wing fluttering lasted for up to 4 secs and generated a low-pitched audible sound. This behavior is believed to be sexual, mainly with regard to mate attraction in some acridid species (Uvarov, 1977).

Five replicates were done for the control and the two doses of pentanoic acid for each of the three groups of locusts, immature males, and mature males and females. A replicate consisted of five insects. Each insect was observed individually in the glass jar only once and was not reused. The number of insects performing a given behavior was recorded and the percentage overall mean of the replicates

calculated for each group. For statistical analysis, data were transformed into arcsine and subjected to a multivariate analysis of variance (MANOVA, $\alpha = 0.05$; SAS Institute, 2000) to determine the dose effects on the behavior of the three groups of locusts. Differences between mean number of insects performing a behavioral activity for the different treatments were determined using paired Student's *t* test.

RESULTS

Gas chromatographic profiles of extracts of CKGs mainly included short-chain aliphatic acids including pentanoic (**1**), hexanoic (**2**), heptanoic (**3**), octanoic (**4**), nonanoic (**5**), and decanoic (**6**) (Figure 1). Pentanoic acid was present in

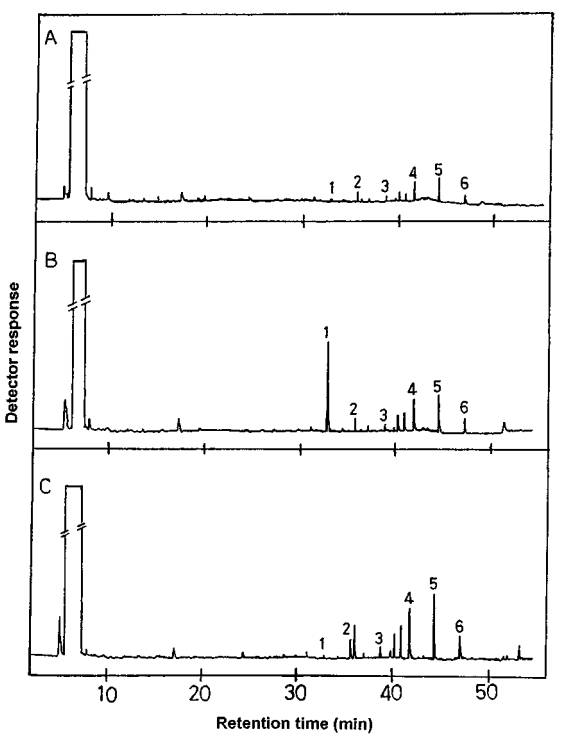


FIG. 1. Representative gas chromatograms of dichloromethane extracts of Comstock-Kellog glands of gregarious female desert locust ($N = 15$) of ages (A) 1–3 days, (B) 14–16 days, and (C) 20–22 days old after final molt showing the identified compounds: pentanoic acid (**1**), hexanoic acid (**2**), heptanoic acid (**3**), octanoic acid (**4**), nonanoic acid (**5**), and decanoic acid (**6**).

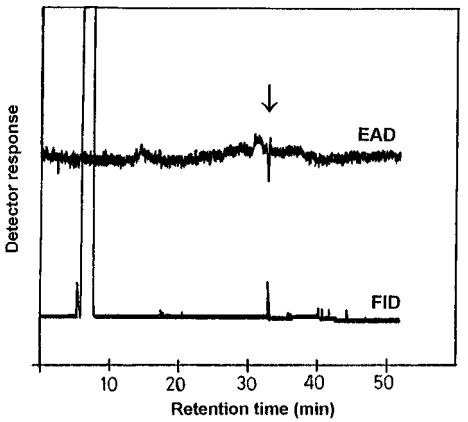


FIG. 2. Computer recorded GC-EAD response from antenna of adult male desert locust, *S. gregaria* to dichloromethane extract of Comstock-Kellog glands of conspecific adult females 14–16 days old after fledging (as in Figure 1). Compound **1**, identified to be pentanoic acid, was EAG-active (arrow).

moderately high amounts in CKG extracts from insects that were 14–16 days old, whereas in younger and older females, only trace amounts were detected. In GC-EAD tests, only pentanoic acid in volatiles from glands of female locusts that were 14–16 days old was EAG active (Figure 2).

In the glass jar bioassay, mature male locusts tended to behave differently (MANOVA, $P < 0.001$) from either the mature females or the immature males when exposed to pentanoic acid. The mature males exhibited strong precopulatory behavior when exposed to dispensers impregnated with 0.05 μg ($t = 15.1$, $df = 8$) and 0.1 μg ($t = 31.2$, $df = 8$) of the compound, whereas the immature males and the mature females did not exhibit the precopulatory behavior (Table 1). In addition, the mature adult males seem to have been more alert to other objects in their proximity than the other insects since a significant proportion ($t = 7.65$, $df = 8$; $P < 0.05$) of them reacted to the blank dispenser (control). On the other hand, all three groups of locusts exhibited secondary arrestment (SAR), which was only slightly significant ($t = 2.61$, $df = 8$; $P < 0.05$) between male and female mature adult locusts at 0.1 μg of pentanoic acid and between the immature adult male and mature adults of both sexes in the control. In essence, immature adult males and mature adult female locusts spent more time either moving up and down on the walls of the jar and climbing the wire mesh or, at some intervals, resting at the base of the jar or underneath the top cover.

Stationary wing-fluttering (SWF) behavior was observed in only a small proportion of mature adult male locusts ($t = 8.45$, $df = 8$; $P < 0.05$) that were exposed to the acid compared to the control (Table 1).

TABLE 1. BEHAVIORAL RESPONSES OF DESERT LOCUSTS TO PENTANOIC ACID (PA)[a]

	Activity (% of insects responding)[b]								
	Precopulatory behavior			Secondary arrestment			Stationary wing-fluttering		
Treatment	Im-male	M-male	M-female	Im-male	M-male	M-female	Im-male	M-male	M-female
Control	0^{a}	$8.01 \pm 1.48^{b*}$	0^{e}	4.0 ± 3.8^{p}	$16.0 \pm 1.98^{x*}$	12.0 ± 1.80^{y}	0^{v}	4.0 ± 1.34^{k}	0^{n}
0.05 μg PA	0^{a}	$43.4 \pm 1.51^{c*}$	20.0 ± 1.59^{f}	16.7 ± 6.7^{r}	13.3 ± 1.58^{x} ns	16.0 ± 1.97^{yz}	0^{v}	$6.67 \pm 1.58^{km*}$	0^{n}
0.10 μg PA	0^{a}	$73.0 \pm 1.86^{d**}$	0^{e}	16.0 ± 1.73^{r}	14.0 ± 1.52^{x}	$18.8 \pm 1.93^{z*}$	0^{v}	$10.0 \pm 1.58^{m*}$	0^{n}

[a] Values are mean $\pm$ SE. Sexually immature adult male, Im-male; mature adult male, M-male; and mature adult female, M-female.

[b] Two-tailed t test for comparison between means: Differences between groups of locusts (rows) for each treatment; *significant ($P < 0.05$, $df = 8$), **highly significant ($P < 0.001$, $df = 8$), and ns = not significant. Differences between treatments for each group (columns) in each behavior activity with the same letter were not significant ($P > 0.05$, $df = 8$). $N = 5$ replicates with five locusts in each.

DISCUSSION

Our study suggests that pentanoic acid functions as a female-produced pheromone that elicits sexual behavior in male *S. gregaria*. There were variations in the relative amounts of pentanoic acid in the gland extracts. The maximum production of the acid was detected in 14- to 16-day-old females, which coincided with their sexual maturity. Under the rearing conditions in our insectary, a small proportion ($\leq$5% in a batch) of female locusts start mating when they were 12 days old after the final molt (fledging). The bulk of females ($\cong$95%) mate 13–15 days after fledging, and eggs are laid one to two days thereafter. In adult male desert locust, the onset of production of phenylacetonitrile, which is the key component of the aggregation pheromone, coincides with their sexual maturation (Mahamat et al., 1993; Deng et al., 1996). Thus, in addition to enlargement of oocytes in gregarious female locusts (Assad et al., 1997a,b), production of pentanoic acid may also increase at the onset of sexual maturity. Furthermore, production of pentanoic acid may influence mate recognition and mating. This is supported by the results of this study, which demonstrate that mature adult male locusts but not immature adult males respond to the odor of pentanoic acid with precopulatory behavior and stationary wing-fluttering when exposed to the acid.

Pentanoic acid, either on its own or in mixtures with other compounds, plays an important chemical ecological role in the behavioral repertoire of a number of insects. It is a sex pheromone for the wireworm, *Limonius californicus* (Mannerheim) (Jacobson et al., 1968) and an attractant for a dipteran *Olcella parva* (Adams) (Hibbard et al., 1997). A mixture of pentanoic acid and hexanoic acid has been used as a standard attractant for the adult rose chafer *Macrodactyla subspinosus* (Williams et al., 1990), and with phenylethanol, the blend is a lure for the onion flies *Delia antiqua* and *D. platura* (Ishikawa et al., 1984).

The results of the glass jar assays further showed that mature male locusts are more sensitive than immature males and mature females to the presence of innate objects in their surroundings. However, SAR was not largely influenced by the presence of pentanoic acid in the three groups. As suggested above, this was a behavioral reaction of the insects to the presence of the dispenser since, in its absence, locusts kept climbing up and down the wire mesh and/or walls of the glass jar.

Stationary wing-fluttering was exclusively observed in mature adult male locusts and was influenced by pentanoic acid, although not as markedly as PCB. There were also indications that there may be an optimum age during which most mature adult males perform this behavior. This behavioral activity is similar to, but more vigorous than, the stridulation of both wings that produces the “short sounds” in a male locust prior to jumping upon a female conspecific (Loher, 1959). However, despite wing-fluttering being a widespread phenomenon in acridids, its relation to sexual behavior has not been rigorously established (Uvarov, 1977).

In some cases, it is not clear whether it is the visual or acoustic or both components of the wing-fluttering that are important. In other insect species, for example the noctuids, wing-fluttering (fanning) may serve to create air movements that influence diffusion of a sex pheromone (Baker and Cardé, 1985). Thus, wing-fluttering in *S. gregaria* males may play a similar role since it has been documented that female *S. gregaria* are stimulated by a volatile substance that is produced by mature adult male conspecifics (Uvarov, 1966).

In summary, this is the first report to identify volatile chemicals from CKGs of gregarious mature adult females of the desert locust. The chemical signal was found to stimulate precopulatory behavior in conspecific mature adult males. The signal was more prominent in sexually mature females than in immature and older ones. However, it is possible that there may be other components of lower polarity that contribute to the specificity of the signal. Follow-up research will focus on identifying any other component(s) of the signal and determining whether the production is influenced by the phase status of the desert locust.

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PERIPHERAL CODING OF SEX PHEROMONE AND A BEHAVIORAL ANTAGONIST IN THE JAPANESE BEETLE, *Popillia japonica*

ALEXANDER A. NIKONOV[1] and WALTER S. LEAL[1,2,*]

[1]*National Institute of Sericultural and Enotomological Science*
1-2 Ohwashi, Tsukuba Science City
Ibaraki 305-8634 Japan

[2]*Department of Entomology*
University of California Davis
Davis, California 95616

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Abstract—Male antennae of the Japanese beetle, *Popillia japonica*, possess olfactory receptor neurons (ORNs) cocompartmentalized in the same sensilla placodea, one tuned to the sex pheromone, (*R*)-japonilure, and the other to the detection of a behavioral antagonist, (*S*)-japonilure. In-depth electrophysiological experiments revealed mutual inhibitory and synergistic effects in ORNs stimulated simultaneously with the two semiochemicals. The olfactory system of *P. japonica* exhibited a remarkable ability to discriminate completely coincident strands of pheromone and behavioral antagonist from strands of the two semiochemicals temporally isolated (by 1.5–3 msec). The mutual inhibition was reflected mainly by the delay of onset or total lack of spikes and by the significant increase in the rise time of potentials generated by blends of (*R*)- and (*S*)-japonilure. In contrast, synergist ORNs showed no neural activity (spikes) when stimulated with either the sex pheromone or the behavioral antagonist, but showed clear responses to blends of the two semiochemicals. Evidence for mixture-suppressed responses was observed not only in the Japanese beetle, but also in the Osaka beetle, *Anomala osakana*, and the Oriental beetle, *Exomala orientalis*, thus suggesting that it is a common feature in the sensory physiology of scarab beetles.

Key Words—*Popillia japonica*, *Anomala osakana*, *Exomala orientalis*, Scarabaeidae, peripheral coding of semiochemicals, single sensillum recordings.

* To whom correspondence should be addressed. E-mail: wsleal@ucdavis.edu

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INTRODUCTION

Insects rely heavily on their highly sensitive and selective olfactory systems to perceive the world. Together with the discovery of sex pheromones (Butenandt et al., 1959), electrophysiological studies (Schneider, 1957) first began to reveal details of peripheral sensory physiology. With chemically pure pheromones available, the insect olfactory system became an experimentally favorable model for addressing basic questions in olfaction, given that antennal sensory neurons could be investigated by subjecting antennal preparations to a single stimulus. Experiments based on electroantennogram (Schneider, 1957) and single sensillum recordings (Schneider and Boeckh, 1962) highlighted the inordinate specificity and sensitivity of the insect olfactory system. While minimal structural modifications to pheromone molecules render them inactive (Kaissling, 1987), a single molecule of the native ligand is estimated to be sufficient to activate olfactory neurons in male antennae (Kaissling and Priesner, 1970). Given that the insect olfactory receptor neurons (ORNs) lie beneath a thick cuticle and are tightly enveloped by auxiliary cells, *in vivo* intracellular recordings at the periphery are technically difficult. On the other hand, the groundbreaking development of intracellular recording from insect brains (Matsumoto and Hildebrand, 1981) and optical measurements of glomerular calcium activities (Joerges et al., 1997) have attracted a great deal of attention and have given emphasis to elucidation of the function(s) of the antennal lobe and signal coding. Currently, it is widely accepted that insect antennae are merely "signal transducers" whose ORNs generate action potentials (spikes) that are transmitted directly to the antennal lobe, where primary processing and spatial and temporal coding take place.

In marked contrast to most insect species, females of the Japanese beetle, *P. japonica* (Coleoptera: Scarabaeidae), produce only a single pheromone compound, (*R*,*Z*)-(−)-(decen-1-yl)-octacyclopentan-2-one, (*R*)-japonilure (Tumlinson et al., 1977). The enantiomer of the Japanese beetle pheromone, (*S*)-japonilure, is used as a sex pheromone of the Osaka beetle, *Anomala osakana* (Leal, 1996). These two species share the same habitat in Japan and can detect each other's pheromone (Leal, 1996). This detection occurs within sensilla placodea on the antennae of each species. Each sensilla placodea contains a pair of ORNs. One of these ORNs responds to a species-specific pheromone, and the other responds to the pheromone of the other species (Wojtasek et al., 1998). In this study, we investigated the sensory physiology of scarab beetles by mimicking natural conditions where insects may encounter not only their own pheromone plume, but also the pheromones of other species, including behavioral antagonists. This study reveals that olfactory signals for behavioral attraction (sex pheromone) and olfactory signals for behavioral antagonism ("stop" signal) in the Japanese beetle are processed and encoded in the periphery before transmission to the antennal lobe.

METHODS AND MATERIALS

Insects. Eggs laid by field-collected beetles were transferred to wet sand in ice cream cups. After hatching, the grubs were transferred individually to ice cream cups filled with humus (Leal et al., 1994). Emerged adults were kept at 20°C (*Exomala orientalis*) or 25°C (*P. japonica*), 65–78% relative humidity, and under a 16L:8D photoregime. Adult *P. japonica* were fed on the artificial diet of the silkworm moth, *Bombyx mori* (Nihon Nosan Kogyo, Yokohama, Japan), whereas adult *E. orientalis* were provided with saturated sucrose solution on cotton.

Olfactory Stimuli. (*R,Z*)-(−)-(Decen-1-yl)-oxacyclopentan-2-one, (*R*)-japonilure, (*S,Z*)-(+)-(decen-1-yl)-oxacyclopentan-2-one, (*S*)-japonilure, (*R,Z*)-(−)-(octen-1-yl)-oxacyclopentan-2-one, *R*-buibuilactone, (*S,Z*)-(+)-(octen-1-yl)-oxacyclopentan-2-one, and (*S*)-buibuilactone were synthesized as previously described (Leal, 1999). The enantiomeric purity of these compounds, determined by gas chromatography with a cyclodextrin-based chiral column (Leal, 1991), was >99.6%. (*Z*)-7-Tetradecen-2-one and (*R,Z*)-(7,15)-(+)-hexadecadien-4-olide were prepared according to Leal et al. (1994) and Kuwahara et al. (1997), respectively.

Single Sensillum Recordings. Antennal preparations were obtained with beetles, and lamellae immobilized in microfuge tubes and dental wax, respectively (Nikonov et al., 2001). Extracellular contacts with ORNs were established by the insertion of either glass or tungsten electrodes under a stereomicroscope (300 × magnification), with the reference electrode placed in the abdomen. Sharp and low impedance glass electrodes (2–5 MΩ, tip 2–3 μm) were prepared from borosilicate glass capillaries (Word Precision Instruments, Inc.; M1B150F-4) with a PC-10 micropipet puller (Narishige, Tokyo, Japan). The glass electrodes were filled with a sensillar lymph solution (Kaissling, 1995), pH 6.5, containing (all in mM): glucose, (22.5), NaCl (25), KCl (171.9), KH_2PO4 (9.2), K_2HPO4 (10.8), $MgCl_2$ (3), $CaCl_2$ (1), HCl (1.5). The tungsten electrodes were electronically sharpened in KNO_2 solution. Electrical contacts were established by inserting an electrode through the cuticle adjacent to a single sensillum placodeum. The antennal preparations were continuously flushed with a charcoal-filtered and moistened air stream (0.4 m/sec) through a stimulus delivery glass tube (8 mmID) placed 10 mm away from the antennal preparation. The glass tube had 5 holes (2 mmID) for introducing stimulus pipets; holes were 1 cm apart, with the first and last ones at 5 and 9 cm away from the outlet, respectively. Each test compound was applied to a piece of filter paper (0.5 × 1 cm) that was dried for 10 min and inserted into a Pasteur pipet. Throughout the paper, the amounts of semiochemicals refer to the amount loaded on filter papers rather than the delivered (puffed) material. Stimulation was performed by inserting the tip of a Pasteur pipet into the hole 5 cm away from the outlet of the stimulus delivery glass tube (6 cm away from the preparation). Air was blown for 300 msec through the pipet by a stimulus controller (CD-02/E;

Syntech, Hilversum, The Netherlands). Simultaneous delivery of two stimuli was done by splitting the airflow into two pipets loaded with different compounds. The two pipets were placed in different holes, and the pipet with the first stimulus to be delivered was placed 5 cm away from the outlet. When glass electrodes were used, receptor generator potentials and spikes were recorded simultaneously. The signals were amplified (Nihon Kohden MEZ-8300; Tokyo, Japan) with bandpass filters of 0–300 Hz and 0.3–3.0 KHz for the receptor generator potentials and spikes, respectively. With tungsten electrodes, only spikes were recorded. Data were stored with Axotape 2.0.2 (Axon Instruments, Foster City, California, USA). Data files were displayed on a computer screen and viewed also on a Nihon Kohden memory oscilloscope VC-11 (Tokyo, Japan). Temporal descriptions of receptor potentials and spikes were done manually from the computer screen. One-tailed interrupted analysis (Zacks, 1971; Hudson, 1977) was used to compare the number of action potentials during a 5-sec prestimulus (for an analysis of spontaneous activity) and during (300 msec) and after (700 msec) the odor stimulation. The neural activity prior to exposure (prestimulus period) was compared with that during stimulation, and the t value obtained was compared to the tabled critical value (37%, $P < 0.05$). The results obtained were statistically processed (Zacks, 1971) and represented as diagrams.

In order to investigate the effect of the time interval on the "mutual inhibition," the relationship between the distance from the stimulus delivery and the antennal preparation was experimentally determined. This was done by stimulating mechanical receptors in the antennae ($N = 10$) with air delivered from 6 to 10 cm away from the antennal preparation (Figure 1) and recording the onset of responses. On the basis of the most linear part of the curve (6–9 cm), it was determined that stimuli delivered 1 cm apart were separated by 15 ± 2.7 msec. Considering the airflow (20 ml/sec) and the diameter of the stimulus delivery glass tube (8 mm), the nominal value is 25 msec/cm.

RESULTS

Colocalized ORNs in Pheromone Sensilla (Type A). In this study, a total of 217 sensilla from 49 male Japanese beetles were examined. Mapping of the antennae showed that the majority of the sensilla (68%) are the pheromone-sensitive sensilla placodea (Kim and Leal, 2000). As previously described (Wojtasek et al., 1998), these sensilla possess two ORNs, one tuned to the sex pheromone [(R)-japonilure] and the other responding to the behavioral antagonist [(S)-japonilure]. Although colocalized, these ORNs are readily distinguishable by their spike amplitudes, with the sex pheromone ORNs showing a larger amplitude than the behavioral antagonist ORNs. The dose–response curves for the pheromone detectors showed remarkable enantiomeric specificity and low threshold (1–10 ng). As previously observed (Wojtasek et al., 1998), the behavioral antagonist ORNs

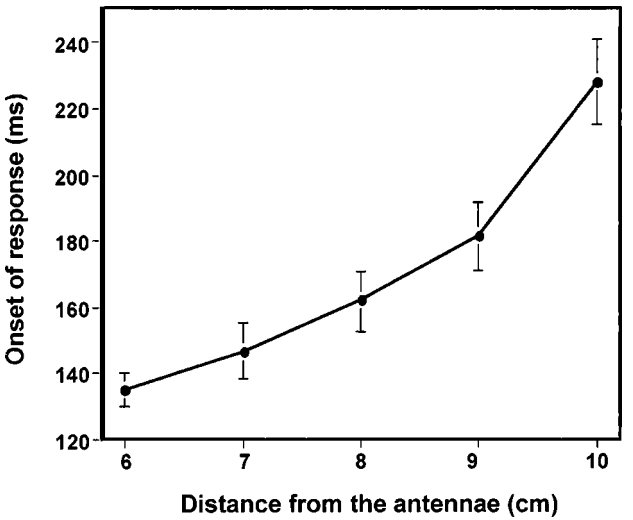


FIG. 1. Relationship between the onset of response ($N = 10$ for each point) in mechanical receptors in the antenna of the Japanese beetle and the distance from the stimulus delivery to the antennal preparation. On the basis of the most linear part of the curve (6–9 cm), stimuli separated by 1 cm were estimated to be temporally isolated by 15 ± 2.7 msec.

were completely silent when challenged with the sex pheromone. However, they were stimulated by the analog lactones (*R*)- and (*S*)-buibuilactone, when subjected to doses higher than their threshold to (*S*)-japonilure (10 ng).

Pheromone Sensilla with Indistinguishable Spike Amplitudes (Type B). In addition to the previously described pheromone sensilla [see above and Wojtasek et al. (1998)], a type of sensilla (14%) stimulated by either (*R*)- or (*S*)-japonilure was also identified. Type B sensilla showed a lower threshold (0.1–1.0 ng) than the other pheromone detectors. Contrary to the large and small spiking ORNs (type A), the amplitudes of the action potentials generated by the sex pheromone and behavioral antagonist in the neurons in type B sensilla were indistinguishable (Figure 2A). The same spike amplitude was observed when the sex pheromone and behavioral antagonist were puffed together or independently.

Sensilla Showing Mutual Inhibition (Type C). Given that the response of the male Japanese beetle is shut down by the behavioral antagonist (Tumlinson et al., 1977), the nervous activity was investigated when sensilla were challenged with a racemic mixture, i.e., the pheromone and behavioral antagonist in equimolar amounts. After observing the firing pattern or the lack of spikes when stimulated with a racemic mixture, the nervous activity generated by the sex pheromone and behavioral antagonist was recorded. Within the pheromone detectors (type A, 68%), a group of sensilla (type C, 36%) was found that showed no spikes when

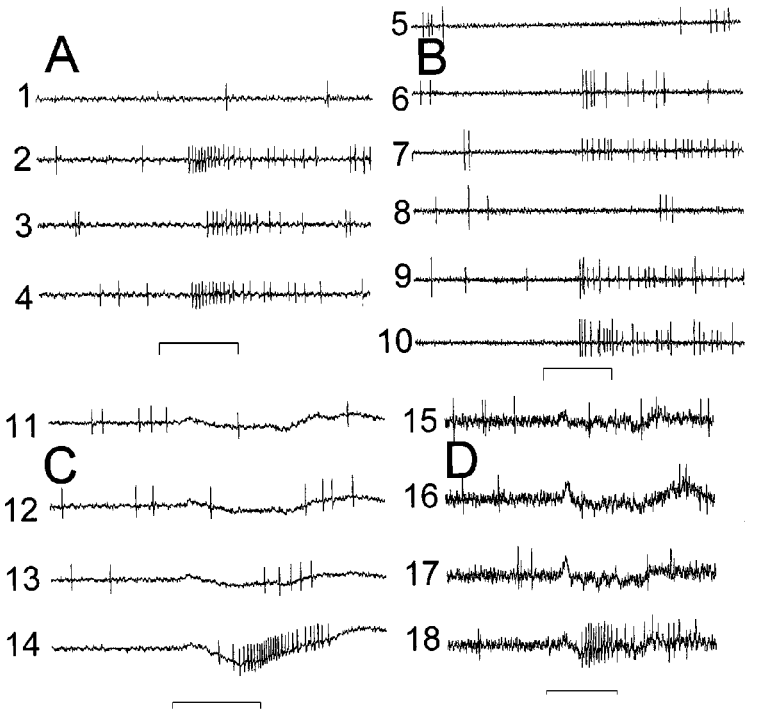


FIG. 2. Response characteristics to the sex pheromone, (*R*)-japonilure, and a behavioral antagonist, (*S*)-japonilure, in olfactory receptor neurons housed in sensilla placodea in the antennae of the Japanese beetle. (A) Recording from type B sensillum with ORNs stimulated by both semiochemicals isolated, or blended. Trace 1, air; 2, (*R*)-japonilure, 0.1 ng; 3, (*S*)-japonilure, 0.1 ng; 4, (*R*)- and (*S*)-japonilure, 0.1 ng of each. (B) Recording from a type C sensillum with larger and smaller spiking ORNs stimulated by the sex pheromone and the behavioral antagonist, respectively. The two neurons were mutually inhibited with blends of the two semiochemicals if their strands were coincident in time. Trace 5, air; 6, (*R*)-japonilure, 100 ng; 7, (*S*)-japonilure, 100 ng; 8, (*R*)- and (*S*)-japonilure, 100 ng of each; 9, (*S*)- and (*R*)-japonilure, (100 ng of each, separated by 60 msec; 10, (*R*)- and (*S*)-japonilure, 100 ng of each, separated by 60 msec. (C) Recording from type D sensillum showing synergist ORNs. Trace 11, air; 12, (*S*)-japonilure, 0.1 ng; 13, (*R*)-japonilure, 0.1 ng; 4, (*R*)- and (*S*)-japonilure, 0.1 ng of each. (D) Recording from the same sensillum in D after excising the antennae. Trace 15, air; 16, (*S*)-japonilure, 0.1 ng; 17, (*R*)-japonilure, 0.1 ng; 18, (*R*)- and (*S*)-japonilure, 0.1 ng of each. These recordings were obtained with tungsten electrodes, and the same patterns shown here were observed at least three times for each type of sensillum after offering the same set of stimuli randomly. The experiments were also replicated with different individuals ($N = 120$). The same experiments were repeated with glass electrodes by recording not only spikes, but also slow potentials. The lines below each set of recordings show the stimulus duration (300 msec).

challenged with a racemic mixture of japonilure (Figure 2B8). These sensilla had two ORNs, one tuned to the sex pheromone (Figure 2B, trace 6) and the other stimulated by the behavioral antagonist (Figure 2B, trace 7). Their dose–curve responses were indistinguishable from those of previously identified pheromone detectors (type A). When challenged with the racemic mixture, most of the type C sensilla (70.6%) showed a complete loss of activity, and the onset of the spikes was tremendously delayed. The other sensilla showed activity for the *R* ORNs, but the spike frequency decreased significantly and the onset of spikes was delayed. Mutual inhibition was observed when the two semiochemicals were delivered at the same time (racemic mixture), but when they were temporally isolated, the cells fired (Figure 2B, traces 9 and 10). This was done by delivering the two semiochemicals at 60-msec intervals. When the sex pheromone was delivered first, the nervous activity was initiated by the large spiking neurons (pheromone detectors) followed by the smaller ones (Figure 2B, trace 10), and when the behavioral antagonist was puffed first, the *S* ORNs responded with lower frequency, but the *R* ORNs showed only a few spikes (Figure 2B, trace 9).

Neuronal responses from the two ORNs were observed when the two stimuli were delivered at 60-, 45-, 30-, or 15-msec intervals either with the sex pheromone or behavioral antagonist delivered first. Mutual inhibition was not observed even when the two stimuli (sex pheromone and behavioral antagonist) were delivered from two pipets with the tips in close proximity, i.e., separated by ca. 1–2 mm (ca. 1.5–3 msec).

Next, the question of whether the ratio of the two semiochemicals is important for the mutual inhibition was considered. Neuronal activity was suppressed when the sensilla were challenged with racemic (1:1) mixtures of the two semiochemicals in the range of 10–10,000 ng. Mutual inhibition was observed with 100:1 and 10:1 mixtures, either with (*S*)- or (*R*)-japonilure being the major constituent. These data were particularly unambiguous when the tests were done in doses higher than the threshold response for the behavioral antagonist ORN (10 ng). At lower doses, the inhibition was not complete, but the onset of response was delayed and the frequency of spikes decreased ($P < 0.05$).

Sensilla Detecting Mixtures (Type D). Another type of sensilla fired only when stimulated with a mixture of the sex pheromone and behavioral antagonist (Figure 2C), but was silent when challenged with the same dose of either the sex pheromone or behavioral antagonist. Type D sensilla (9%) showed high sensitivity (threshold 0.1 ng), but their performances were restricted to the range of 0.1–10 ng. When challenged with higher doses, they responded to individual enantiomers, with (*R*)-japonilure stimulating the ORNs with slightly larger spike neurons. These sensilla showed the same synergistic effect when challenged with the two enantiomers in 10:1 ratios, particularly when at lower doses (0.1–1 ng). Because of the slight difference in spike amplitudes, it was not possible to determine whether the responses were due to one of the neurons or to a combination.

In order to investigate whether the mutual inhibitory and synergistic effects were related to any feedback from the brain, analogous experiments were conducted with excised antennae. The results (shown in Figure 2D) were obtained with the same antenna used for the experiments shown in Figure 2C. Although there was an increase in the noise, the responses with intact insect and excised antennae showed the same profile. This was the case with both mutual inhibition (data not shown) and the synergistic effect (Figure 2D).

Other Types of Sensilla. A small number of sensilla (2%) showed ORNs tuned either to the sex pheromone or the behavioral antagonist. Although the other enantiomer (antipode) did not elicit spikes, it generated a slow potential. Both the *R*- and *S*-type ORNs failed to respond to racemic mixtures (no spikes were observed), but they generated slow potentials. Another type of sensilla (1%) showed a basal firing (natural spikes), generated a slow potential when stimulated with (*R*)- and (*S*)-japonilure, but never generated spikes.

Mutual Inhibition in Other Species. The influence of semiochemical blends on the firing patterns of pheromone-detecting neurons was investigated in other scarab species. Preliminary experiments with field-collected ($N = 10$) Osaka beetle, *A. osakana*, showed a similar type of mutual inhibition. However, a laboratory colony of this species could not be established, and an in-depth investigation was not performed. As previously demonstrated (Wojtasek et al., 1998), the pheromone detectors in *A. osakana* were stimulated by (*S*)-japonilure and generated large spikes, whereas (*R*)-japonilure elicited nervous activity (with smaller spike amplitude) in colocalized neurons. The mutual inhibition was observed in the tested range (100–1000 ng) either with racemic or 10:1 mixtures.

In the Oriental beetle ($N = 25$), the sex pheromone, (*Z*)-7-tetradecen-2-one (Leal et al., 1994), stimulated a large spiking neuron (Figure 3A). In the same sensilla, there is a second neuron (smaller spiking amplitude), which was activated by the sex pheromone of another scarab species, *Heptophylla picea*, (*R*,*Z*)-7,15-hexadecadien-4-olide (Leal et al., 1996) (Figure 3B). A 1:1 mixture of the two pheromones (100 ng of each) had a major influence in the firing pattern of the pheromone detector in the Oriental beetle (Figure 3C). The onset of pheromone spikes was dramatically delayed, and the frequency was significantly lower. Conversely, the onset of spikes of the neuron detecting the interspecific pheromone was shorter, and the frequency was significantly higher. Furthermore, the slow potential generated by the mixture was quite different from the slow potential elicited by the two compounds independently, in particular the rise phase potential was significantly slower.

Electrical Signals Characteristics. An in-depth investigation of type C sensilla in the Japanese beetle was carried out to determine the effect of mutual inhibition on the electrical signals generated by the receptor cells. The parameters (Figure 3) characterizing the signature of the nervous activity (Kaissling, 1987), i.e., the onset of slow potential (t_0), the onset of spikes (t_1), the rise time (t_2), and the fall of the

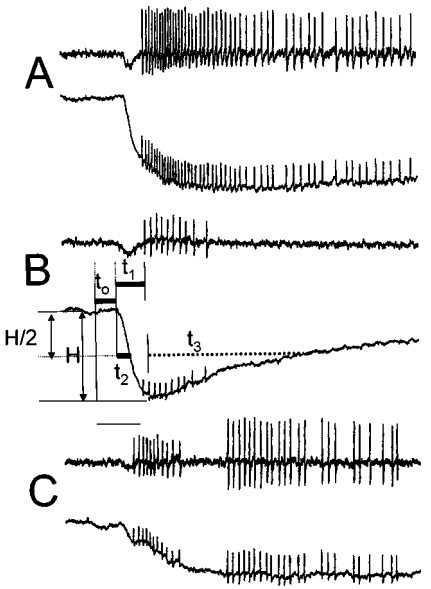


FIG. 3. Single sensillum recordings from pheromone-detecting sensilla placodea in the Oriental beetle obtained with a glass electrode. Two ORNs differing in their spike amplitudes are housed in these sensilla. They were stimulated by the sex pheromone of the Oriental beetle, (*Z*)-7-tetradecen-2-one (A, 100 ng), and the sex pheromone of the yellowish elongate chafer, *Heptophylla picea*, i.e., (*R*,*Z*)-7,15-hexadecadien-4-olide (B, 100 ng). There was mutual inhibition of the two ORNs by a blend of the two semiochemicals (C, 100 ng of each). Experiments were replicated with the same individual at least three times and with different individuals ($N = 9$). The lines below the recording in B show the stimulus duration (300 msec). Parameters used to evaluate the effect of mutual inhibition on the characteristics of the signals: t_0, onset of slow potential; t_1, onset of spikes; t_2, rise phase time; t_3, fall of the slow potential, and H, amplitude of the slow potential.

slow potential (t_3), were analyzed. The most striking influence was the onset of spikes (t_1, Table 1; Figure 2B, trace 8). Spikes generated by 10 and 100 ng of a racemic mixture were significantly delayed. In various experiments ($N > 80$), it was not possible to determine accurately if the spikes were delayed or the activity was completely lost. This was because the spike frequency was so low and the onset so delayed (e.g., Fig 2B, trace 8) that it was impossible to distinguish them from basal firing (spontaneous nervous activity).

Although there was no significant difference in the onset of the slow potential when the sensilla were subjected to individual compounds or a racemic mixture (t_0, Table 1), the rise time was significantly delayed with higher concentrations of the racemic mixture (t_2, Table 1). Contrary to the slow potential generated

TABLE 1. EFFECT OF SIMULTANEOUS STIMULATION WITH SEX PHEROMONE AND BEHAVIORAL ANTAGONIST ON ELECTRICAL SIGNALS GENERATED BY PHEROMONE-DETECTING SENSILLA (TYPE C) IN JAPANESE BEETLE ($N = 16$)

Stimulus	Amount (ng)	t_0 (ms)	t_1 (ms)	t_2 (ms)	t_3 (ms)
R	10	95 ± 9	95 ± 11	70 ± 7	280 ± 30
S	10	90 ± 9	50 ± 7	65 ± 8	220 ± 24
$R + S$	10	101 ± 13	180 ± 24	50 ± 6	201 ± 20
R	100	90 ± 10	58 ± 6	50 ± 8	217 ± 21
S	100	91 ± 11	33 ± 7	67 ± 7	305 ± 27
$R + S$	100	83 ± 12	150 ± 16 (S) 201 ± 18 (R)	151 ± 14	205 ± 25

either with the sex pheromone or behavioral antagonist, the rise potential with the racemic mixture showed two phases, a slow rise phase followed by a fast rise phase of the slow potential. The general effect was a significant delay of the rise phase as compared to individual semiochemicals. This delay in the rise phase of the slow potential was also observed in the silent sensilla (see above) that showed slow potential but no spikes.

DISCUSSION

Research was conducted to determine how the peripheral sensory system in the Japanese beetle perceives the environment, particularly with regard to the detection of the sex pheromone, (*R*)-japonilure, and a behavioral antagonist, (*S*)-japonilure. The latter is a sex pheromone of the Osaka beetle. These two species share the same habitat in parts of the Japanese archipelago, and the pheromone of one species is detected as a behavioral antagonist (stop signal) by the other (allospecies) (Tumlinson et al., 1977; Leal, 1996, 1998). Therefore, under natural conditions, these beetles encounter not only their own sex pheromone but also blends of the two semiochemicals. An in-depth investigation of the peripheral sensory physiology in the Japanese beetle antennae that mimicked natural conditions revealed an interesting feature of its olfactory system. Coding of the sex pheromone and behavioral antagonist can be achieved with the peripheral sensory system given that the antennae possess: (1) detectors activated only by a mixture of the two semiochemicals, (2) detectors specific for each enantiomer, and (3) detectors with neuronal activity decreased when they encounter a mixture of the two compounds. Therefore, the nervous activity sent to the antennal lobe already contains a signature characterizing the presence/absence of sex pheromone and/or behavioral antagonist.

Previously, synergist neurons have been identified in the antennal lobes in the brain of the earworm moth *Helicoverpa zea* (Christensen et al., 1991). The neurons

in the antennal lobes of *H. zea* were silent to the major pheromone constituent, (*Z*)-11-hexadecenal, and a behavioral antagonist, (*Z*)-9-hexadecenal, produced by the sympatric species, *Heliothis virescens*. However, the neurons responded only when the two semiochemicals were offered together (Christensen et al., 1991). A synergistic effect at the sensory neuronal level has been observed in the tobacco cutworm moth, *Spodoptera litura* (Aihara and Shibuya, 1977). Stimulation with a mixture (9:1) of the major sex pheromone constituent, (*Z,E*)-9,11-tetradecadienyl acetate, and a second constituent, (*Z,E*)-9,12-tetradecadienyl acetate, increased the frequency of spikes more than the major constituent alone (Aihara and Shibuya, 1977).

Although mutually inhibitory neurons have never been observed in insect antennae, there are a few examples of mixture interactions occurring among insect ORNs that would alter the information at the periphery before reaching the antennal lobe (Todd et al., 1992). In the summerfruit tortrix moth, *Adoxophyes orana*, the responses of an ORN to separately applied (*Z*)-11-tetradecenyl acetate concentrations are higher than those to the same concentrations of this compound when mixed with the major pheromone constituent, (*Z*)-9-tetradecenyl acetate (Den Otter, 1977). An interaction between plant odor and pheromone perception has been documented in the small ermine moth, *Yponomeuta evonymellus*. In this species, geraniol inhibits response to the sex pheromone, (*Z*)-11-tetradecenyl acetate (van der Pers et al., 1980). Inhibition of pheromone receptor activity by plant chemicals has also been found in the male wild silkmoth, *Antheraea pernyi* (Schneider et al., 1964) and silkworm moth, *B. mori* (Kaissling et al., 1989). In the cabbage looper moth, *Trichoplusia ni*, the binary mixture containing the major pheromone constituent, (*Z*)-7-dodecenyl acetate, and a behavioral antagonist, (*Z*)-7-dodecenol, did not elicit significant deviation from additivity in either olfactory receptor neuron. However, addition of dodecyl acetate to this blend resulted in a mixture that significantly reduced the observed response of both ORNs below that expected by the additive model (O'Connell, 1985; O'Connell et al., 1986). Responses to the major pheromone constituent, (*Z,Z,Z*)-3,6,9-nonadecatriene, in a type of sensilla in the mottled umber, *Erannis defoliaria*, were inhibited by the second constituent, (*Z,Z*)-3,9-*cis*-6,7-epoxy-nonadecadiene, which produces hyperpolarization (Hansson et al., 1990). A comprehensive study of the sensory physiology in normal and mutant *T. ni* showed that a single minor component could apparently hyperpolarize one cell while depolarizing another cell within the same sensillum (Todd et al., 1992). It has been also observed in the maxillary palp of *Drosophila melanogaster* that a particular odor can excite one neuron and inhibit another and that a particular neuron can be excited by one odor and inhibited by another (de Bruyne et al., 1999). However, mutual inhibition at the periphery is reported here for the first time.

It is conceivable that mixture or blend interactions in insect antennae may have been overlooked previously because the widely studied lepidopteran species have complex pheromone systems that may obscure this feature. Clearly, the

behavioral significance of the two semiochemicals in the biology of the Japanese beetle facilitated our studies. However, peripheral coding has not been observed in bark beetles, for which pheromones and behavioral antagonists are also clearly identified. Earlier studies indicate that the majority of the ORNs in the antennae of the bark beetle *Ips pini* were activated either by the aggregation pheromone, ipsdienol, or the behavioral antagonist, ipsenol (Mustaparta et al., 1977). Simultaneous stimulation with the two semiochemicals showed that ipsenol had no inhibitory effect on the receptor response to ipsdienol, leading to the conclusion that behavioral inhibition results from central integration of the information from separate receptor cells rather than a peripheral event (Mustaparta et al., 1977). Further studies with pure enantiomers and racemic mixtures indicate that there is no synergistic or inhibitory interaction of the enantiomers on the receptor cells in *I. pini* and *I. paraconfusus* (Mustaparta et al., 1980). Whether or not peripheral coding of sex pheromones and behavioral antagonists is a general feature in insect olfaction must await further studies, but the findings presented here suggest that it is the case in more than one species of scarab beetles. In this study, mutual inhibition was observed not only in the Japanese beetle, but also in the Osaka beetle, *A. osakana*, and the Oriental beetle, *E. orientalis*. A comprehensive investigation in the Japanese beetle sensory physiology showed not only mutual inhibition but also a synergistic effect.

The remarkable fine resolution of the olfactory system in the Japanese beetle allows discrimination of completely overlapped plumes of sex pheromone and behavioral antagonist from those derived from the two semiochemicals temporally isolated (1.5–3 msec). This is in agreement with the evidence in the literature that a behavioral antagonist must be emitted from the same point source as a pheromone to be optimally effective (Witzgall and Priesner, 1991; Liu and Haynes, 1992, 1993). *H. zea*, for example, can distinguish completely coincident strands of pheromone and behavioral antagonist from those whose strands are separated by only 1 mm [3 msec at 40 cm/sec wind (Baker et al., 1998)]. It has been pointed out that the ability to discriminate their conspecific pheromone strands from the strand of a nonconspecific female could have fitness advantages to males. This fine resolution would allow males to not be erroneously deterred by the presence of antagonistic compounds in a plume and to successfully locate and mate with their own females (Todd and Baker, 1999). Such fine resolution would likely be easily achieved at the periphery rather than by central processing of information. This might be the reason for cocompartmentalized ORNs that detect pheromones and behavioral antagonists (Baker et al., 1998; Todd and Baker, 1999).

It is not known unequivocally what happens to the activities of antennal neurons farther down the antennal nerve where there could potentially be interactive effects due to gap junctions or other interaxonal phenomena (Todd and Baker, 1999). The first line of evidence for mutual inhibition and synergist ORNs in scarab beetles prompted us to study the ultrastructure of sensilla placodea in

the Japanese beetle (Kim and Leal, 2000). Following assignment of pheromone-detecting sensilla placoda by single sensillum recording, their ultrastructures were studied. Each pheromone sensillum had two ORNs (Kim and Leal, unpublished) in agreement with the electrophysiological findings of two ORNs characterized by the spike amplitudes. The observation of two ORNs in all pheromone-detecting sensilla placodea traced by transmission electronic microscopy (TEM) led us to conclude that all types of sensilla have two ORNs, even when the discrimination on the basis of spike amplitude is not clear (types A and C).

The internal morphology of the pheromone-detecting sensillum placodeum in the Japanese beetle is similar to that of sensilla trichodea of moth antennae, except for near-cuticle structures. The outer and inner dendritic segments of ORNs are enclosed by three enveloping cells: thecogen, trichogen, and tormogen. High-magnification micrographs reveal that there are desmosomal contacts between the inner dendritic segments and the thecogen cells (Kim and Leal, 2000). If these desmosomal contacts function like gap junctions, electrical interaction between one ORN tuned to pheromone and a behavioral antagonist detecting ORN may lead to the peripheral mutual inhibition observed in the Japanese beetle.

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EFFECT OF *ent*-LABDANE DITERPENES FROM POTAMOGETONACEAE ON *Selenastrum capricornutum* AND OTHER AQUATIC ORGANISMS

TIZIANA CANGIANO,[1] MARINA DELLAGRECA,[2] ANTONIO FIORENTINO,[1,*] MARINA ISIDORI,[1] PIETRO MONACO,[1] and ARMANDO ZARRELLI[2]

[1]*Dipartimento di Scienze della Vita*
Seconda Università di Napoli
via Vivaldi 43, 81100 Caserta, Italy

[2]*Dipartimento di Chimica Organica e Biochimica*
Università Federico II
Complesso Universitario Monte S. Angelo
via Cinthia 4, I-80126 Napoli, Italy

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Abstract—Twenty *ent*-labdane diterpenes, isolated from the aquatic plants *Ruppia maritima* and *Potamogeton natans*, were tested to detect their effects on aquatic organisms from different trophic levels. Toxicity tests were performed on aquatic producers (the alga *Selenastrum capricornutum*), and consumers including a rotifer (*Brachionus calyciflorus*), a cladoceran crustacean (*Daphnia magna*), and two anostracan crustaceans (*Thamnocephalus platyurus* and *Artemia salina*). Furano-*ent*-labdanes exhibited high toxicity toward all of these organisms. 15,16-Epoxy-12(*S*)-hydroxy-8(17),13(16),14-*ent*-labdatrien-20,19-olide had a high toxicity only toward the algae and the rotifers. It was inactive for the crustaceans.

Key Words—Potamogetonaceae, *Potamogeton natans*, *Ruppia maritima*, diterpenes, *ent*-labdane, aquatic organisms activity, antialgal activity, toxicity testing.

INTRODUCTION

The potential role of allelochemicals in agriculture and forestry has been extensively reported (Waller, 1987). This is in contrast to the chemical interactions

* To whom correspondence should be addressed. E-mail: antonio.fiorentino@unina2.it

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between vegetal organisms in aquatic systems that have not been much studied, despite evidence of potential allelopathic interactions.

Schreiter (1928) was the first to hypothesize that aquatic plants could interfere with phytoplankton, citing a case in which phytoplankton growth decreased if there was a high presence of aquatic plants. Hasler and Jones (1949) observed a reduction of the algal population in ponds where some species of Potamogetonaceae were present. They confirmed their observation through an experiment using densely planted and plant-free silo-ponds, finding that microalgal growth was significantly less in the densely planted ponds than in the plant-free ponds. Fitzgerald (1969) indicated that some species of aquatic weeds could control the algal growth. Many other experiments also identified interactions between hydrophytae and microalgae (Kogan and Chinnova, 1972; Brammer, 1979; Godmaire and Planas, 1983), although allelopathy was not mentioned. Hootsmans and Blindow (1994) affirmed that the allelopathic limitation of algal growth could not be excluded, but suggested that the allelopathic effects might vary with regard to place and time and that they might be dependent on nutrient concentration and the relative age of the organisms.

The ability of some natural products to inhibit in vitro development of microalgae has been reported by our research group in recent years (DellaGreca et al., 1996, 1998), and we have shown that bioactive products isolated from *Pistia stratiotes* are released into the environment (DellaGreca et al., 1999).

In connection with a study of the allelopathic interactions among aquatic plants distributed in the Mediterranean basin and microalgae, and on the basis of the previously reported observations of Hasler and Jones (1949), we examined two species of Potamogetonaceae, which grow in the Volturno, the largest river of southern Italy. The first one, *Potamogeton natans*, commonly known as water tongue, is a freshwater species, while *Ruppia maritima*, commonly known as sea hay, lives at the mouth of the river in brackish waters. The phytochemical study of these plants led to the isolation of *ent*-labdane diterpenes, identified previously on the basis of their spectroscopic properties (DellaGreca et al., 2000; Cangiano et al., 2001).

The aim of this study was to determine the effect of the diterpene metabolites from Potamogetonaceae, not only on algae, but also on aquatic species from various taxonomic levels to provide a wider range of ecotoxicity information (Rand, 1995).

METHODS AND MATERIALS

General Experimental Procedures. NMR spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a Bruker AC 400 spectrometer in $CDCl_3$ or CD_3OD solutions, at 27°C. Proton-detected heteronuclear correlations were measured using HMQC (optimized for $^{1}J_{HC} = 145$ Hz) and HMBC (optimized for $^{1}J_{HC} = 7$ Hz). Optical rotations were measured on a Perkin-Elmer 343 polarimeter. IR spectra were determined in $CHCl_3$ solutions on a FT-IR Perkin-Elmer 1740 spectrometer. UV spectra were obtained on a Perkin-Elmer Lambda 7 spectrophotometer in

EtOH solutions. EI-MS spectra were obtained with a Kratos MS 80 apparatus. FAB mass spectra were obtained with a Kratos VG ZAB 2SE. The HPLC apparatus consisted of a pump (Shimadzu LC-10AD), a refractive index detector (Shimadzu RID-10A), and a Shimadzu Chromatopac C-R6A recorder. Preparative HPLC was performed using RP-8 (Luna 10 μm, 250 $\times$ 10 mm ID, Phenomenex), SiO_2 (Maxsil 10 μm, 250 $\times$ 10 mm ID, Phenomenex), or RP-18 (Kromasil 10 μm, 250 $\times$ 10 mm ID, Phenomenex) columns. Analytical TLC was performed on Merck Kieselgel 60 F_{254} or RP-18 F_{254} plates with 0.2 mm layer thickness. Spots were visualized by UV light or by spraying with H_2SO_4–AcOH–H_2O (1:20:4). The plates were then heated for 5 min at 110°C. Preparative TLC was performed on Merck Kieselgel 60 F_{254} plates, with 0.5 or 1 mm film thickness. Flash column chromatography (FCC) was performed on Merck Kieselgel 60 (230–400 mesh) at medium pressure. Column chromatography (CC) was performed on Merck Kieselgel 60 (70–240 mesh) or on Sephadex LH-20 (Pharmacia).

Plant Material. Plants of *Potamogeton natans* and *Ruppia maritima* were collected from the Volturno River, Italy, and identified by Professor Antonino Pollio at the Università Federico II of Naples. Voucher specimens (HERBNAPF065) were deposited at the Dipartimento di Biologia Vegetale of the Università Federico II of Naples.

Extraction and Isolation of Diterpenes from R. maritima (Figure 1). Air-dried plants (10 kg) were sequentially and exhaustively extracted with light petrol,

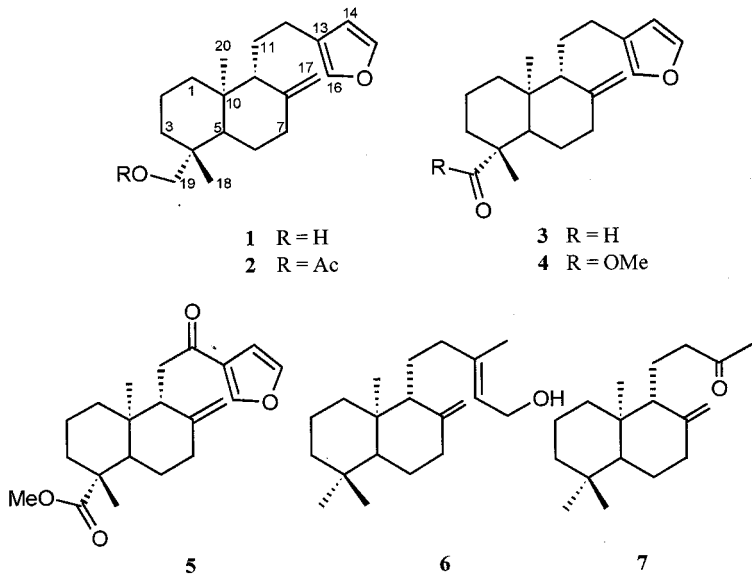


FIG. 1. *ENT*-Labdane-diterpenes isolated from *Ruppia maritima*.

EtOAc, and MeOH. The light petrol extract (36 g) was chromatographed on neutral Al_2O_3 (grade III), and elution with hexane–Et_2O (19:1) gave fractions A–D. Fraction A was purified on Sephadex LH-20 [hexane–$CHCl_3$–MeOH (4:1:1)] to give compound **1** (23 mg). Fraction B was chromatographed on silica gel eluting with hexane–$CHCl_3$ (4:1) to give pure **3** (31 mg) and **4** (37 mg). Fraction C was chromatographed on silica gel eluting with hexane–benzene mixtures. Hexane–benzene (49:1) gave crude **6**, which was purified by preparative TLC [hexane–benzene (9:1)]. The fraction eluted with hexane–benzene (9:1) was chromatographed by RP-18 HPLC [MeOH–MeCN (19:1)] to give compounds **2** (8 mg) and **7** (35 mg). Fraction D was chromatographed on Sephadex LH-20 with hexane–$CHCl_3$–MeOH (4:1:1) to give compound **5** (8 mg), which was purified by RP-18 HPLC [MeOH–MeCN (19:1)].

Extraction and Isolation of Diterpenes from P. natans (Figure 2). The air-dried and powdered plant material (13 kg) was sequentially and exhaustively extracted with light petrol, EtOAc, and MeOH. The light petrol extract (66 g) was

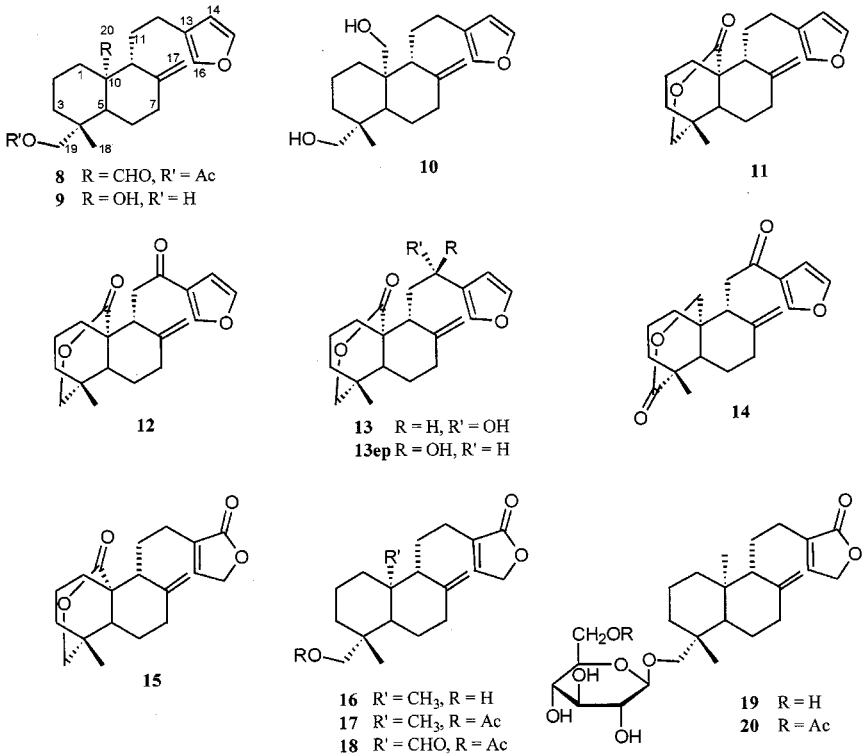


FIG. 2. *ENT*-Labdane-diterpenes isolated from *Potamogeton natans*.

chromatographed on silica gel eluting with hexane and EtOAc solutions to give fractions A and B. Fraction A, eluted with hexane–EtOAc (19:1), was chromatographed using hexane–EtOAc (17:3) as eluent to give pure compounds **8** (57 mg) and **11** (125 mg). Fraction B, eluted with hexane–EtOAc (17:3), was filtered on Sephadex LH-20 using hexane–$CHCl_3$–MeOH (3:1:1) as eluent; fractions 12–38 were purified by HPLC, using a preparative silica gel column and a mixture of hexane–*i*Pr_2O (3:1) as eluent, and gave pure **17** (15 mg) and **18** (11 mg); fractions 56–89 were purified by preparative TLC, using a mixture of benzene–EtOAc (19:1) as eluent, to give pure **12** (85 mg) and **14** (4 mg). The EtOAc extract (79 g) was chromatographed on silica gel eluting with hexane and EtOAc solutions to give fractions C–E. Fraction C, eluted with hexane–EtOAc (4:1), was chromatographed on Sephadex LH-20 with hexane–$CHCl_3$–MeOH (3:1:1). The elute obtained was purified by HPLC, using a preparative RP-8 column and a mixture of MeOH–H_2O (4:1) as eluent, to give pure **9** (11 mg) and **13** (35 mg). Fraction D, eluted with hexane–EtOAc (2:1), was chromatographed on preparative TLC [$CHCl_3$–EtOAc (3:1)] to give compound **10** (31 mg). Fraction E, eluted with hexane–EtOAc (3:1), was chromatographed on preparative TLC [benzene–EtOAc (3:2)] to give compounds **15** (13 mg) and **16** (51 mg). The methanolic extract (120 g) was treated with H_2O and EtOAc. The aqueous layer, concentrated to 500 ml, was chromatographed on Amberlite XAD-4. The fraction eluted with MeOH was chromatographed on Sephadex LH-20 [MeOH–H_2O (1:1)] to give a mixture of glycosides, which were separated on preparative RP-18 eluting with MeOH–H_2O (4:1) to give pure **19** (21 mg) and **20** (8 mg).

*Preparation of **13** and **13ep***. $NaBH_4$ (8 mg) was added to a solution of **12** (30 mg) in MeOH (2.5 ml), and the reaction mixture was stirred for 5 hr at room temperature. The hydride in excess was broken with AcOH (2 drops). The mixture was then directly purified by preparative TLC with $CHCl_3$–EtOAc (17:3) to give, besides the unreacted **12** (11 mg), compound **13** (5 mg) and 12(*S*)-hydroxy-15,16-epoxy-8(17),13(16),14-*ent*-labdatrien-20,19-olide (**13ep**, 11 mg).

Preparation of (R)- *and* (S)-*MTPA Esters of **13***. (*S*)-(+)-MTPA-Cl (5 μl) was added to a solution of pure **13** (1.5 mg) in dry pyridine (50 μl). After 4 hr at room temperature under magnetic stirring, ethyl ether (5 ml) and water (5 ml) were added to the reaction mixture. The organic layer gave a crude ester, which was purified by preparative TLC eluting with hexane–EtOAc (5:1). The ester (*R*)-MTPA had ^{1}HNMR ($CDCl_3$, 400 MHz) δ: 6.45 (1H, s, H-14), 5.95 (1H, d, $J = 10.5$ Hz, H-12), 5.01 (1H, s, H-17), 4.69 (1H, s, H-17), 4.18 (1H, dd, $J = 1.5$, 11.6 Hz, H-19), 4.01 (1H, d, $J = 11.5$ Hz, H-19), 2.65 (1H, m, H-11), 2.11 (1H, m, H-11), 0.90 (3H, s, H-18). (*S*)-MTPA of **13** was prepared by the same procedure. ^{1}HNMR ($CDCl_3$, 400 MHz) δ: 6.38 (1H, s, H-14), 5.91 (1H, dd, $J = 4.7$, 10.5 Hz, H-12), 5.02 (1H, s, H-17), 4.76 (1H, s, H-17), 4.14 (1H, dd, $J = 1.5$, 11.5 Hz, H-19), 3.98 (1H, d, $J = 11.5$ Hz, H-19), 2.75 (1H, m, H-11), 2.26 (1H, m, H-11), 0.89 (3H, s, H-18).

Preparation of (R)- *and* (S)-*MTPA Ester of* ***13ep***. The procedure used for the MTPA esters of **13** was also used for the preparation of (*R*)-MTPA and (*S*)-MTPA of **13ep**. The (*R*)-MTPA ester had ^{1}HNMR ($CDCl_3$, 400 MHz) δ: 6.29 (1H, s, H-14), 6.03 (1H, dd, $J = 4.7$, 10.5Hz, H-12), 4.99 (2H, s, H-17), 4.16 (1H, dd, $J = 1.5$, 11.5 Hz, H-19), 4.00 (1H, d, $J = 11.5$ Hz, H-19), 2.95 (1H, m, H-11), 2.23 (1H, m, H-11), 0.89 (3H, s, H-18). The ester (*S*)-MTPA had ^{1}HNMR ($CDCl_3$, 400 MHz) δ 6.41 (1H, s, H-14), 6.02 (1H, dd, $J = 4.7$, 10.5Hz, H-12), 5.01 (1H, s, H-17), 4.99 (1H, s, H-17), 4.16 (1H, dd, $J = 11.5$, 1.5 Hz, H-19), 4.00 (1H, d, $J = 11.5$ Hz, H-19), 2.85 (1H, m, H-11), 2.20 (1H, m, H-11), 0.88 (3H, s, H-18).

Toxicity Testing. Microbiotests independent of the culturing and/or maintenance of live stocks of test organisms, known as Toxkits™ (Creasel, Deinze, Belgium) were selected for this study (Persoone and Janssen, 1993; Persoone et al., 1993; Persoone, 1998a,b). The Toxkits for freshwater were used in accordance with conditions and media prescribed by OECD (1994), ISO (1987), AFNOR (1983), and US EPA (1991), following the standard operational procedure described for the respective Toxkit tests. There were: (1) the Algaltoxkit F (Creasel, 1996), a 0-72 hr algal growth inhibition assay with *Selenastrum capricornutum*; (2) the Rotoxkit F (Creasel, 1992), a 24 hr rotifer mortality test (test species: *Brachionus calyciflorus*); (3) the Thamnotoxkit F (Creasel, 1995), a 24 hr crustacean mortality test (test species: *Thamnocephalus platyurus*, anostraca); and (4) the Daphtoxkit F (Creasel, 2000), a 24 hr crustacean immobilization test (test species: *Daphnia magna*, cladocera).

The toxicity tests were conducted by exposing the organisms to solutions of varying concentrations of single chemicals of high purity as reported in our previous studies (DellaGreca et al., 2000, 2001b). In the present study, to the test battery we added a further bioassay with the anostracan *Artemia salina*, a marine species, to detect the impact of metabolites released by *R. maritima* and *P. natans* in proximity of the mouth of the river where salinity is substantially higher. The crustacean toxicity test to assess the mortality of *A. salina* was conducted by hatching these anostraca from cysts incubated for two days at 25°C in synthetic seawater in the dark. The solutions were prepared in five toxicant concentrations (twofold concentration increments) for each compound, each with three replicates of 10 animals in disposable multiwell plates (1 ml per well). After 24 hr in a 25°C incubator in the dark (Creasel, 1990), the number of dead crustaceans was counted.

Data Analysis. Raw data for all bioassays, except the algal test, were analyzed by using the Toxcalc Toxicity Data Analysis and Database Software, ver. 5.0 (Tidepool Scientific Software). Point estimations were calculated by concentration response regression with Probit Analysis as first choice and Trimmed Spearman-Karber as second, if Probit was not possible. If the raw data did not allow the respective LC/EC_{50} to be calculated at the highest tested concentrations, then these values were determined by a direct graphical method. Raw test data from Algaltoxkit were analyzed by a Microsoft Excel 5.0 program tailored for this test.

The algal median growth inhibition was estimated by integrating the mean values from time 0 to 72 hr (area under the curve). Inhibition (percentage) values were reported against log-transformed data of concentrations (micromoles per liter) to evaluate the slope, the trend of regression and the IC_{50} value.

RESULTS AND DISCUSSION

The structures of the 20 *ent*-labdane diterpenes tested in this research are reported in Figures 1 and 2. Plants of *R. maritima* and *P. natans* were air-dried and extracted with light petrol, ethyl acetate, and methanol. Chromatographic processes (CC, TLC, and HPLC) of the extracts of the first plant led to isolation of seven *ent*-labdanes (**1–7**). Five (**1–5**) of them have a furano ring in the skeleton, and one (**7**) is a *nor*-bis-diterpene. Compounds **1** and **4** had already been isolated from *Psiadia altissima* (Canonica et al., 1969) and *Daniella oliveri* (Haeuser and Lombard, 1961), respectively, while all the other compounds were isolated here for the first time from *R. maritima*.

Chromatographic processes of the extracts of the water tongue afforded six furano-*ent*-labdanes (**8**, **10**–**14**), six γ-lactone-*ent*-labdanes (**15**–**20**), two of them as glucosides (**19** and **20**), and one *nor*-diterpene (**9**) (Cangiano et al., 2001; DellaGreca et al., 2001a). All the furano diterpenes (**8**–**14**) were isolated for the first time from *P. natans*, except compound **11**, which had already been isolated by Smith et al. (1976) and is known as potamogetonin. To establish the configuration at C-12 of the compound **13**, 12-oxopotamogetonin (**12**) was reduced with $NaBH_4$ to give the natural **13** and its epimer **13ep** at C-12. Mosher's method (Dale and Mosher, 1973; Othani et al., 1991) was applied to both the products. The (*R*)-MPTA and (*S*)-MTPA esters showed differences in the chemical shifts in agreement with the *S* configuration for **13** and, consequently, the *R* configuration for its epimer (**13ep**). The lactonediterpenes (**15**–**20**) were isolated for the first time in *P. natans*, except for compounds **16** and **19**, known as andrograpanine (Fujita et al., 1984) and neoandrographolide (Chan et al., 1971), respectively, which were previously isolated from *Andrographys paniculata*.

All the natural products (**1**–**20**), and the synthetic **13ep**, were assayed in standard water against the green alga *Selenastrum capricornutum*, one of the organisms recommended for the standard algal assay procedure developed for aquatic systems (Pipe and Shubert, 1984) to measure the potentially adverse effect of chemicals on the aquatic environment. The toxicity is reported as IC_{50}, the inhibiting concentration that reduces algal growth by 50% (Table 1).

The tests revealed that the presence of the furano group in the molecule is important for the antialgal activity. Among all the furano diterpenes, the most toxic was compound **1**, showing an IC_{50} 0.8 μmol/liter. The free hydroxyl group at C-19 seems to be important for such activity; in fact, the acetylation of the C-19 position (compare **1** and **2**) increases the IC_{50} value (13.62 μmol/liter), retaining

TABLE 1. EFFECTS OF DITERPENES ON *Selenastrum capricornutum*, REPORTED AS IC_{50} WITH 95% CONFIDENCE LIMITS[a]

Compounds	IC_{50} (μmol/liter)	Compounds	IC_{50} (μmol/liter)
1	0.8 (0.43–1.41)	**11**	28.58 (23.85–36.63)
2	13.62 (10.95–16.96)	**12**	60.89 (57.8–62.8)
3	7.57 (6.90–8.33)	**13**	4.40 (3.11–6.21)
4	ND	**13ep**	ND
5	1.45 (1.07–2.0)	**14**	ND
6	ND	**15**	44.16 (25.11–61.9)
7	ND	**16**	217.13 (200.4–221.8)
8	58.27 (37.94–89.49)	**17**	696.25 (680.7–703.4)
9	2.84 (1.11–3.02)	**18**	NR
10	18.45 (13.73–24.81)	**19**	80.54 (68.52–94.70)
		20	53.70 (34.27–61.91)

[a] ND = not determined at highest concentration assayed; NR = no relation dose–response.

a dose–response relationship in compound **2,** but diminishing the activity. Compound **3** is a growth stimulant at low concentrations, and the toxic effect was revealed only by increasing the concentrations ($IC_{50} = 7.57$ μmol/liter). The toxicity of **4** was significant for concentrations higher than 9.5 μmol/liter, and its IC_{50} could not be found at the highest tested concentration (45 μmol/liter). The carbonyl group at C-19 decreases the activity of the chemicals unless another carbonyl is present at C-12 (compound **5**). Furthermore, the lactone ring between the C-19 and C-20 decreases the inhibitory effect. In fact, the IC_{50} of potamogetonin **11** was 28.58 μmol/liter, while the 12-oxo derivative **12** was biostimulating up to a concentration of 25.85 μmol/liter and became inhibiting at the highest concentration (60.89 μmol/liter). The activity becomes significant again because of the presence of a hydroxyl group at C-12, as shown by compound **13** with an IC_{50} of 4.40 μmol/liter. It is important to note that epimer **13ep** acts in a completely different way. It is only slightly active (20% inhibition) at the highest tested concentration (136.4 μmol/liter), indicating that the configuration of carbon 12 is fundamental to the biological activity. On the other hand, it is reported that the algal growth effect is dependent on the stereochemistry of the substrates (DellaGreca et al., 1990). The absence of the furano group (compounds **6** and **7**) reduces the activity of the chemicals considerably, and the oxidation of the heterocyclic ring to γ-lactone decreases the toxicity, as is evident from the comparison of compounds **11**, **16**, **17**, and **18** with the analogs **15**, **1**, **2**, and **8**. Furthermore, statistical analysis performed with Student's t test (comparison between averages) shows that the furano compounds are more toxic than lactones ($P < 0.01$).

The glucosylation of the lactone diterpenes (**19** and **20**) causes biostimulation at the low concentrations. These results can be explained by the fact that detoxification often occurs through glucosylation (Harborne, 1977).

TABLE 2. EFFECTS OF FURANO DITERPENES ON AQUATIC ORGANISMS REPORTED AS L(E)C_{50} WITH 95% CONFIDENCE LIMITS

Compounds	*D. magna* (EC_{50})[a]	*T. platyurus* (LC_{50})[a]	*B. calyciflorus* (LC_{50})[a]	*A. salina* (LC_{50})[a]
1	157.8 (113.0–248.0)	0.84 (0.76–0.94)	66.59 (59.54–74.47)	ND
2	ND	126.1 (87.85–143.1)	6.60 (5.23–7.85)	ND
3	ND	ND	4.54 (1.64–7.73)	ND
8	19.8 (9.78–30.11)	9.41 (5.84–15.17)	56.11 (44.63–74.86)	ND
10	47.17 (40.41–55.06)	ND	74.87 (65.38–85.75)	ND
11	14.59 (11.27–18.88)	23.56 (14.68–37.86)	36.97 (30.32–45.22)	ND
12	35.0 (23.04–53.20)	74.30 (56.19–98.23)	ND	ND
13	ND	ND	1.48 (1.21–1.82)	ND
13ep	ND	ND	ND	ND

[a] In micromoles per liter. ND = not determined at highest concentration assayed.

To evaluate the impact of these diterpenes on other aquatic organisms, present in the same ecosystems as the investigated Potamogetonaceae, we tested the most active and abundant chemicals on consumers, including a rotifer (*Brachionus calyciflorus*), a crustacean cladoceran (*Daphnia magna*), and two anostracans (*Thamnocephalus platyurus* and *Artemia salina*). Acute toxicity of labdane diterpenes was detected by exposing the organisms to solutions of varying concentrations and counting the number of dead (*T. platyurus*, *B. calyciflorus*, and *A. salina*) or immobile (*D. magna*) organisms. We report the toxicity as LC_{50} or EC_{50}, the lethal or immobilizing concentrations that affect 50% of the exposed organisms (Table 2).

The activity of the diterpenes on *D. magna* is less than that on *S. capricornutum*. Compounds **2**, **3**, **13**, and **13ep** are completely inactive at the highest concentrations tested. The carbonyl group at carbon 20 and the absence of an oxygenated function at the carbon 12 (compounds **8** and **11**) seems to enhance the toxicity.

In contrast to the previously mentioned species, compounds **1** and **8** were very active on *T. platyurus*. The LC_{50} value of the first compound (0.84 μmol/liter) was comparable with its IC_{50} value for *S. capricornutum* (0.80 μmol/liter). Compounds **3**, **10**, **13**, and **13ep** were inactive at the tested concentrations, while *ent*-labdanes **2** and **12** showed high values of LC_{50}.

B. calyciflorus was sensitive to most of the tested compounds. Only compounds **12** and **13ep** were inactive for this invertebrate. The most toxic for rotifers was **13**, showing a LC_{50} of 1.48 μmol/liter. Diterpenes **2** and **3** also showed a high toxicity, with LC_{50} values lower than 7 μmol/liter. *A. salina*, already known as a resistant organism (Persoone, 1998), was not affected by exposure to these chemicals.

It should be noted that the synthetic **13ep** was active on all species tested, while its natural epimer **13** showed high toxicity values only for algae and rotifers. Treatment of **12** with $NaBH_4$ afforded the alcohol **13ep** in two-fold yield compared

to the epimer **13**. The absence of **13ep** in the plant allows one to suppose the involvement of an appropriate enzymatic system in the biosynthesis of **13** and a plausible specific role of this metabolite.

Except for compound **13ep**, no one compound was active or inactive toward all the organisms. Compound **3** was inactive for *D. magna* and *T. platyurus*, but had low of LC_{50} and IC_{50} values for *B. calyciflorus* and *S. capricornutum*. Compound **1** was highly toxic toward *T. platyurus* and algae, while showing high LC_{50} and EC_{50} values for *B. calyciflorus* and *D. magna*, respectively.

The biological activity of diterpenes is thoroughly reported in the literature. Singh et al. (1999) reviewed the biological activity of labdane diterpenes from 1987 to 1997. The antibacterial, antifungal, antiinflammatory, cytotoxic, and other biological activities detected in the labdane diterpenes have been widely discussed in relation to chemical structures. In recent years, the role of diterpenes in allelopathy has been supported (Macias et al., 1999, 2000), but so far the antialgal activity of these compounds has not been reported. The only exception is geniculol, an irregular diterpenoid isolated from an ecdophytic fungus of the *Geniculosporium* genus, which inhibits the growth of the green alga *Chlorella fusca* (König et al., 1999). The toxicity of the labdanes on other aquatic organisms also is but slightly known, and only the brine shrimp (*Artemia salina*) has been extensively employed in bioassays (Gray et al., 1998).

One of the factors supporting the use of algae and invertebrates in bioassays is their niche in aquatic ecosystems. If these organisms are adversely affected by a toxicant, then the surrounding ecosystem may also feel the effects, either directly or indirectly, from the lack of a food source. Algae and invertebrates occupy a low trophic level, and their disturbance may cause a chain effect throughout the ecosystem.

The results obtained from the phytochemical study of *P. natans* and *R. maritima* suggest that the high concentrations of the bioactive diterpenes found in these plants interfere with other aquatic organisms, thus playing a role in the equilibrium of the aquatic systems.

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DOES THE ODOR FROM SPONGES OF THE GENUS *Ircinia* PROTECT THEM FROM FISH PREDATORS?

JOSEPH R. PAWLIK,[1,*] GREG MCFALL,[1] and SVEN ZEA[2]

[1]*Biological Sciences and Center for Marine Science Research*
University of North Carolina at Wilmington
Wilmington, North Carolina 28403-3297

[2]*Universidad Nacional de Colombia (Departamento de Biología)*
INVEMAR, Cerro Punta de Betín
AA 10-16, Santa Marta, Colombia

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Abstract—Caribbean sponges of the genus *Ircinia* contain high concentrations of linear furanosesterterpene tetronic acids (FTAs) and produce and exude low-molecular-weight volatile compounds (e.g., dimethyl sulfide, methyl isocyanide, methyl isothiocyanate) that give these sponges their characteristic unpleasant garlic odor. It has recently been suggested that FTAs are unlikely to function as antipredatory chemical defenses, and this function may instead be attributed to bioactive volatiles. We tested crude organic extracts and purified fractions isolated from *Ircinia campana*, *I. felix*, and *I. strobilina* at naturally occurring concentrations in laboratory and field feeding assays to determine their palatability to generalist fish predators. We also used a qualitative technique to test the crude volatile fraction from *I. felix* and *I. strobilina* and dimethylsulfide in laboratory feeding assays. Crude organic extracts of all three species deterred feeding of fishes in both aquarium and field experiments. Bioassay-directed fractionation resulted in the isolation of the FTA fraction as the sole active fraction of the nonvolatile crude extract for each species, and further assays of subfractions suggested that feeding deterrent activity is shared by the FTAs. FTAs deterred fish feeding in aquarium assays at concentrations as low as 0.5 mg/ml (fraction B, variabilin), while the natural concentrations of combined FTA fractions were >5.0 mg/ml for all three species. In contrast, natural mixtures of volatiles transferred from sponge tissue to food pellets and pure dimethylsulfide incorporated into food pellets were readily eaten by fish in aquarium assays. Although FTAs may play other ecological roles in *Ircinia* spp., these compounds are effective as defenses against potential predatory fishes. Volatile compounds may serve other defensive functions (e.g., antimicrobial, antifouling) but do not appear to provide a defense against fish predators.

* To whom correspondence should be addressed. E-mail: PawlikJ@UNCWil.edu

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INTRODUCTION

Sponges produce a wide variety of marine natural products that often exhibit potent activity in pharmacological assays (Faulkner, 2000, and previous reviews cited therein). Yet, the roles that these metabolites play in sponge biology remain largely unknown. Because secondary metabolites in sponges are structurally complex and frequently present in high concentrations, it has been presumed their synthesis occurs at some metabolic cost to the organism, and for this cost, they should provide some benefit (Paul, 1992; Pawlik, 1993; McClintock and Baker, 2001). Sponge secondary metabolites may act to inhibit biofouling and overgrowth (Henrikson and Pawlik, 1995, 1996), inhibit the growth of microorganisms (Newbold et al., 1999; Zea et al., 1999), prevent damage caused by UV radiation (Paul, 1992), or act as allelopathic agents (Sullivan et al., 1983; Porter and Targett, 1988; Engel and Pawlik, 2000), but the most commonly hypothesized function is that of predator deterrence (Pawlik, 1993; Pawlik et al., 1995; Chanas et al., 1996; McClintock, 1997; Wilson et al., 1999; Waddell and Pawlik, 2000a,b).

In a previous study, we surveyed the crude organic extracts of 71 species of Caribbean sponges (Pawlik et al., 1995) and found that 63.4% contained metabolites that were unpalatable to the generalist predatory fish *Thalassoma bifasciatum* in aquarium assays. Among the least palatable were extracts from sponges of the genus *Ircinia*. Three species of *Ircinia—I. felix, I. strobilina*, and *I. campana*—are commonly found on tropical coral reefs, grassbeds, and in mangroves throughout the Caribbean (Zea, 1987; Schmahl, 1991).

Sponges of the genus *Ircinia* are well known for their strong, unpleasant garlic odor, which has recently been traced to a mixture of low-molecular-weight, volatile nitrogen- or sulfur-containing compounds, including dimethylsufide, methyl isocyanide, and methyl isothiocyanate (Duque et al., 2001). These volatile compounds exhibit antimicrobial activity in laboratory assays (Duque et al., 2001). In addition, furanosesterterpene tetronic acids (FTAs) have been isolated from *Ircinia* spp. (Cimino et al., 1972; Martínez et al., 1997) (Figure 1). These compounds reportedly have a variety of biological activities in various assay systems, including antimicrobial activity and the inhibition of Ca^{2+} transport (Beveridge et al., 1995), but their potential ecological functions have never been experimentally addressed.

Recently, Zea et al. (1999) reported that concentrations of FTAs in *Ircinia felix* were lower in the sponge surface than in internal tissues, greater in sponges found or transplanted to areas of lower light intensities, and greater in sponges that had been intentionally injured. Moreover, they reported that, when injured, *I. felix*

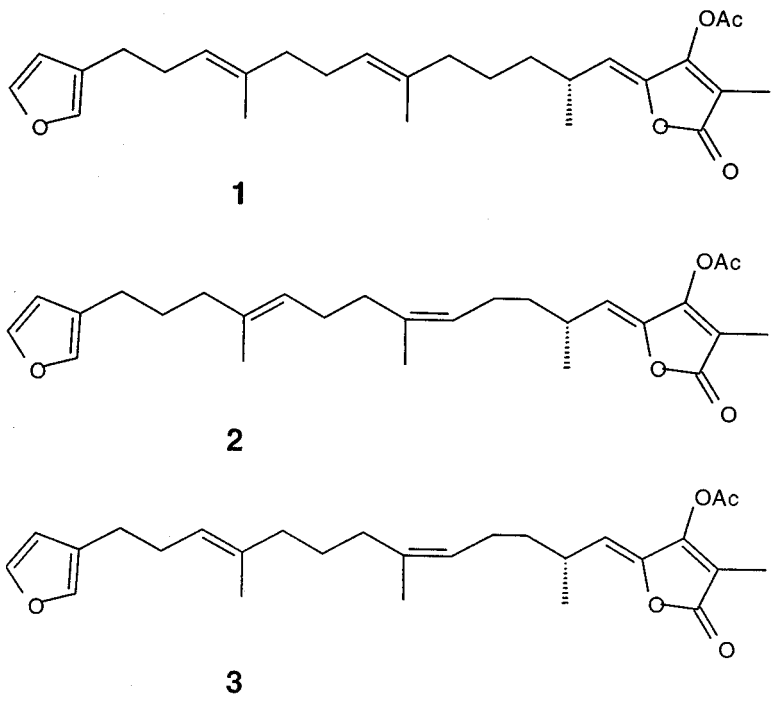


FIG. 1. Furanosesterterpene tetronic acids (FTAs, shown as acetate esters) from *Ircinia* spp. **1**, variabilin; **2**, strobilinin; **3**, felixinin.

did not release FTAs into the surrounding water column, suggesting that these nonpolar compounds were not involved in external ecological interactions such as chemical defense against potential predators, antifouling, or antiovergrowth (Zea et al., 1999). Instead, they suggested that the odor-causing, volatile compounds were responsible for chemical defense, either by direct action, or at a distance by signaling invasive organisms or potential predators of the presence of FTAs within the sponge tissue (Zea et al., 1999; Duque et al., 2001).

Considering the foregoing, we became interested in the relative antipredatory role of volatiles and FTAs from *Ircinia* spp. Standard techniques for isolating crude organic extracts, such as those used in Pawlik et al. (1995), result in the loss of highly volatile constituents, and to our knowledge, volatile metabolites of sponges have not previously been subjected to feeding assays. Specifically, we wanted to answer these questions: (1) Which nonvolatile components of the crude organic extracts of *Ircinia* spp. are responsible for the antipredatory effects described previously (Pawlik et al., 1995)? (2) Do the odor-causing volatile metabolites of *Ircinia* spp. also contribute to the antipredatory chemical defenses of these sponges?

METHODS AND MATERIALS

For experiments with nonvolatile crude organic extracts of sponges, samples of *Ircina felix* and *I. strobilina* were collected in August 1995 from reefs, mangroves, and seagrass beds from Acklins Island, Long Island, and Sweetings Cay, Bahamas, at 1–30 m depth. Samples of *I. campana* were collected October 1995 at Rodriguez Cay, Key Largo, Florida, at 1–2 m depth. Replicate specimens were obtained from geographically distinct locations to avoid collection of asexually reproduced clones. Samples were removed from the substrate by cutting the tissue with a sharp knife. Sponges were identified according to Zea (1987). Specimens were extracted immediately or frozen at -20°C until extraction.

Sponge tissue was chopped into cubes, tissue volume was measured by displacement, and the tissue was serially extracted in methanol and a 1:1 mixture of dichloromethane (DCM) and methanol (MeOH) (details in Pawlik et al., 1995; Chanas et al., 1996). The resulting crude extract was resuspended and divided into aliquots that were stored under nitrogen and kept at -20°C until use in aquarium or field assays. To separate compounds based on polar affinity, an aliquot of each crude extract was successively partitioned against water, hexanes, DCM, and *n*-butanol. The four partitions were tested for feeding deterrency in aquarium and field assays (see below).

Aquarium assays indicated that antifeedant activity was present in the hexanes and DCM partitions. Subsequent reverse-phase (C_{18}) thin-layer chromatography (TLC) of all the partitions using 9:1 MeOH–H_2O as the mobile phase revealed that these two partitions contained the same compounds. The hexanes and DCM partitions were combined as a nonpolar partition, and the *n*-butanol and aqueous partitions were likewise combined to form a polar partition, and both combined partitions were subjected to aquarium and field assays.

The combined nonpolar partition was dried and repartitioned between hexanes and acetonitrile. TLC analysis (as above) indicated nearly complete separation of at least two compounds into the acetonitrile repartition, with two pink-stained spots having R_f values of approximately 0.45 and 0.55, respectively. Both repartitions were subjected to aquarium assays, and the acetonitrile repartition was also assayed in the field.

Reverse-phase preparative TLC was used to isolate two fractions (A and B) from the acetonitrile repartition using 7:3 MeOH–H_2O as the mobile phase. Fractions A and B were further purified by using high-performance liquid chromatography (Spherisorb ODS-2 C_{18} column with a mobile phase of 65:34.7:0.03 acetonitrile–water–trifluoroacetic acid). Fraction A was a mixture of compounds, but fraction B was a single metabolite and was subjected to mass spectrometry (HP 5890 series II gas chromatograph coupled with an HP 5971 mass detector) and to ^{1}H and ^{13}C NMR spectroscopy using a 300-MHz Varian model NMR.

For assays of the naturally occurring volatile metabolites from *Ircinia* spp., individual specimens of *I. felix* and *I. strobilina* were collected in July 2000 at Sweetings Cay and Little San Salvador, Bahamas, from 14–18 m depth. For each species, sponge samples were drained of excess seawater and pureed in a blender. The resulting sponge slurry (125 ml) was stirred and slightly heated in a stoppered Erlenmeyer flask through which air was bubbled from an aquarium air pump. The air emerging from the flask was passed by airline tubing through an air-stone in a test tube containing distilled water (20 ml) chilled in a bath of ice in seawater. After several trials, the ratio of 125 ml of sponge slurry to 20 ml of distilled water produced an odor from the distilled water at 25°C that was approximately as strong as that of the slurry from which it was obtained. The distilled water containing the mixture of volatile metabolites was immediately used to make alginic acid food (see below) and subjected to aquarium fish feeding assays. Pure dimethylsulfide, at concentrations equivalent to 1, 10, and 100 times those found in the tissues of *Ircinia felix* was also subjected to aquarium fish feeding assays (Duque et al., 2001) (natural concentration was $\sim$0.3 μg/g of ash-free tissue dry weight, equivalent to 0.29 μl/liter, calculated from an average ash-free weight of 82 mg/ml of sponge; cf. Chanas and Pawlik, 1995).

Aquarium assays using the generalist reef predator, *Thalassoma bifasciatum*, were performed according to the methods detailed in Pawlik et al. (1995). Briefly, crude extracts, partitions, fractions, or pure compounds were incorporated at natural volumetric concentrations (or higher, for fractions A and B and for dimethylsulfide) into a food matrix composed of alginic acid and freeze-dried, powdered squid mantle. The matrix was extruded through a syringe into a 0.25 M solution of $CaCl_2$ to produce a hardened noodle that was then cut into food pellets. Treated and control pellets (lacking addition of extract or compounds) were offered to 10 replicate sets of three fish. A treated pellet was considered deterrent if it was not eaten after three or more attempts to take it into their mouths, by one or more fish or if it was ignored. Control pellets were eaten in all assays, as fish that would not eat control pellets were considered satiated and were not used in assays. For any single assay of 10 replicates, an extract was significantly deterrent if four or more pellets were rejected ($P < 0.043$, Fisher exact test, one-tailed).

Field assays were conducted as described in Chanas and Pawlik (1995) and Chanas et al. (1996) on nonvolatile crude extracts and all partitions of all three *Ircinia* spp. Field assays were conducted on shallow reefs near Key Largo, Florida, in May 1996, or in the Bahamas (most at Sweetings Cay) during July–August 1996 or 1997. Briefly, crude extracts, partitions, or fractions were incorporated at natural volumetric concentrations into a heated food matrix composed of carrageenan and freeze-dried, powdered squid mantle. The heated mixture was then poured into plastic molds crossed by lengths of cotton string that protruded from the ends of the molds. After the matrix had cooled and set, 1.0 $\times$ 0.5 $\times$ 5.0-cm strips were

sliced to size with a razor blade and removed from the mold. For each experiment, 20 treated strips and 20 control strips were prepared. One treatment and one control strip each were tied to a 50-cm length of three-strand nylon rope at a distance of approximately 4 and 12 cm from one end of the rope (the order was haphazard). Twenty ropes were deployed on the reef, with the end of each rope opposite the food strips attached to the substratum by inserting a piece of nonliving substratum through the rope twines. Within 1 hr, the ropes were retrieved and the amount of each strip eaten was recorded as a percentage decrease in the strip length (to the nearest 5%). The Wilcoxon paired-sample test (one-tailed) was employed to analyze the results after excluding pairs for which both control and treatment slices had been either completely eaten, or not eaten at all.

RESULTS

Food pellets containing natural concentrations of crude organic extracts of *Ircinia campana*, *I. felix*, and *I. strobilina* deterred feeding of *Thalassoma bifasciatum* in aquarium assays (Figure 2), and deterred feeding of a natural assemblage of reef fishes (Figure 3). Food pellets containing the water and *n*-butanol (BuOH)

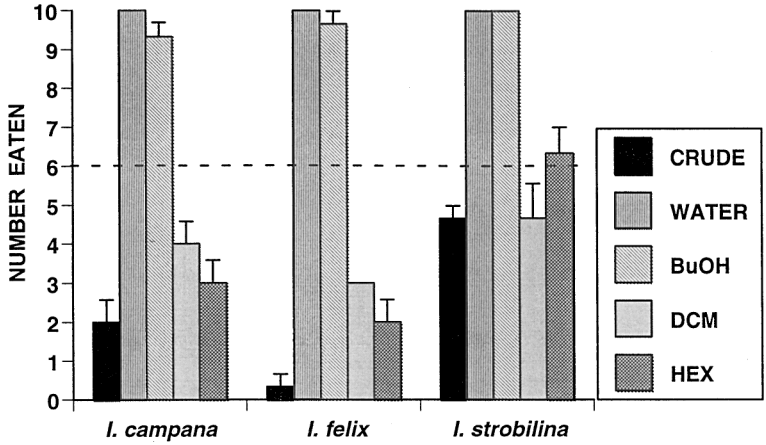


FIG. 2. Aquarium assay. Consumption by *Thalassoma bifasciatum* of food pellets containing natural concentrations of crude extracts and solvent partitions of the crude extracts from three *Ircinia* spp. For each species, assays were replicated with extracts and partitions from 3 sponge samples ($N = 3$: mean + SD is shown). BuOH = butanol partition, DCM = dicholormethane partition, HEX = hexane partition. For each assay, all 10 control food pellets were eaten. For any individual assay, extracts were considered deterrent if the number of pellets eaten was ≤ 6 ($P \leq 0.043$, Fisher exact test) as indicated by the dotted line on the graph.

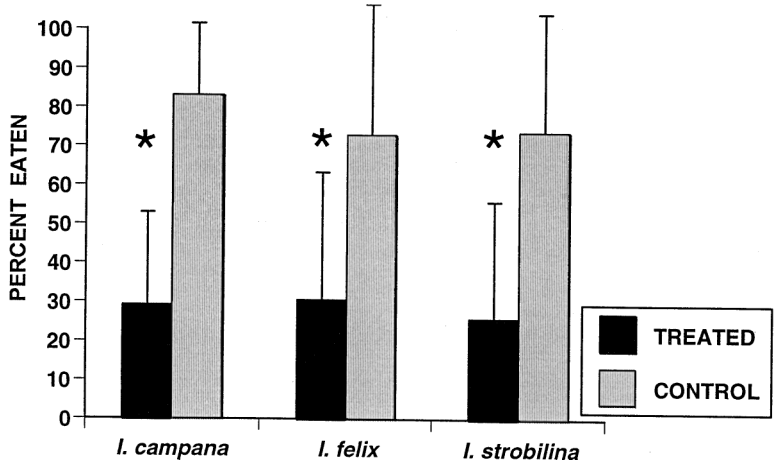


FIG. 3. Field assay. Consumption by reef fishes of paired control food strips and strips containing the natural concentration of the crude organic extracts from three *Ircinia* spp. Mean + 1 SD is indicated. For each assay, 20 pairs of food strips were deployed in the field. $^{*}P < 0.05$, Wilcoxon paired-sample test.

partitions of the crude extracts of all three species were palatable, and the compounds responsible for antifeedant activity were restricted to the dichloromethane and hexanes partitions (Figure 2). Field assays of the combined polar (water and BuOH) partitions and combined nonpolar (DCM and hexanes) partitions confirmed the results of the aquarium assays, with the activity restricted to the nonpolar partitions (Figure 4). Repartitioning of the combined nonpolar partitions between acetonitrile and hexanes further separated the activity: the acetonitrile repartition deterred feeding in field assays for each species, while the reconstituted remainder of the crude extract was not deterrent (Figure 5).

The acetonitrile repartition yielded two spots by TLC analysis, and these were separated and quantified by HPLC into fractions A and B for each of the three species of *Ircinia* (Table 1). Fractions A and B from *Ircinia felix* were subjected to aquarium assays at natural concentrations and at lower concentrations in order to ascertain the minimal effective concentration (Table 2).

Analysis of fractions A and B by HPLC revealed that fraction A was a mixture of compounds, but that fraction B was a single metabolite. Analysis of fraction B by GC-MS yielded a M′ of 429 with a base peak of 73. NMR data revealed the structure of fraction B to be that of the known FTA variabilin (**1**). Fraction A was not further separated or analyzed, but was most likely a mixture of the other FTAs present in the mixture isolated from *Ircinia* spp. (Cimino et al., 1972; Martínez et al., 1997) (Figure 1).

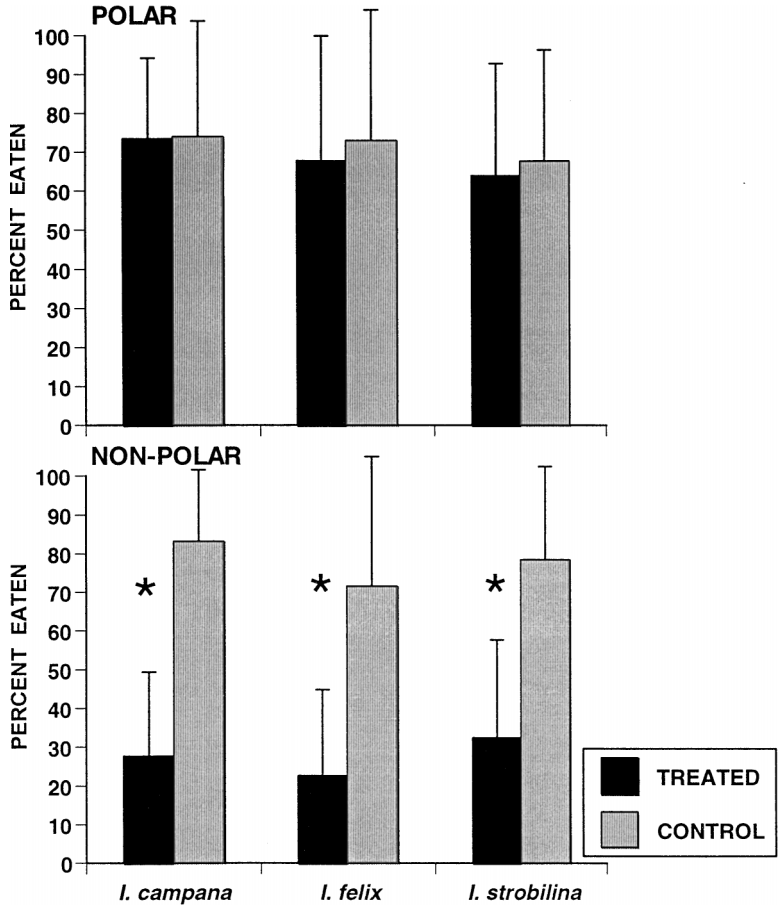


FIG. 4. Field assay. Consumption by reef fishes of paired control food strips and strips containing natural concentrations of recombined polar (water and BuOH) and nonpolar (DCM and hexanes) solvent partions of the crude organic extracts of three *Ircinia* spp. Data presented as in Figure 2.

Food pellets containing roughly natural concentrations of the volatile metabolites from *I. felix* and *I. strobilina* were readily eaten by *Thalassoma bifasciatum* in aquarium assays. Pellets exhibited odor at the same level of intensity as sponge slurry when presented to the fish. Food pellets containing 1, 10, and 100 times the natural concentration of dimethylsulfide as tissue of *Ircinia* spp. were also readily eaten by fish in aquarium assays, although these pellets did not have a noticeable smell at the time of the assays.

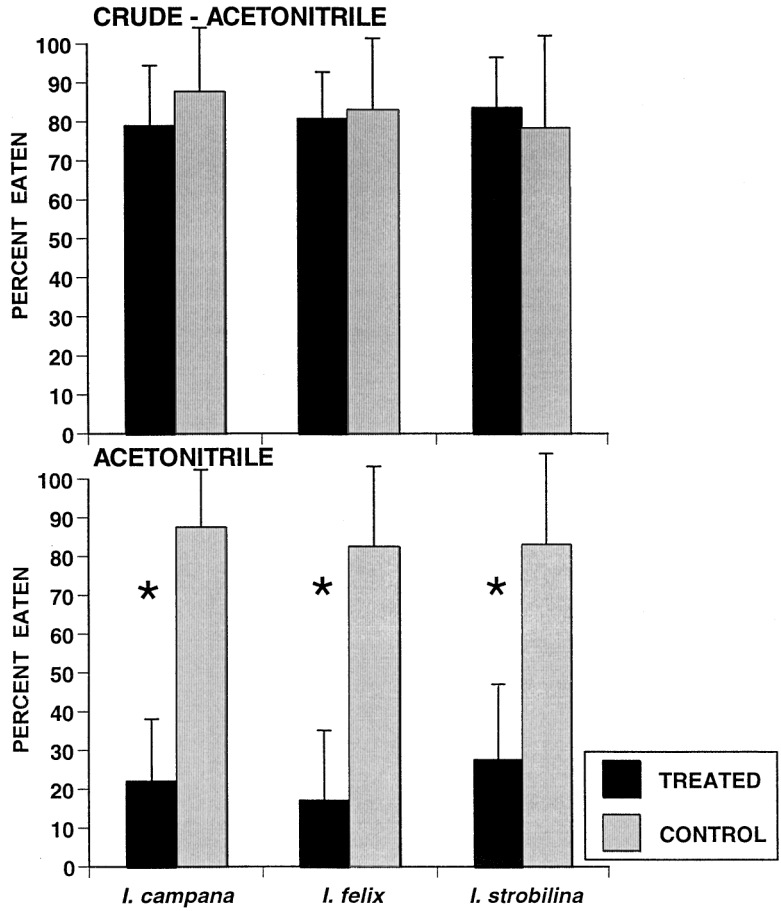


FIG. 5. Field assay. Consumption by reef fishes of paired control food strips and strips containing natural concentrations of the recombined crude extract minus the acetonitrile repartition and the acetonitrile repartition from the crude organic extracts of three *Ircinia* spp. Data presented as in Figure 2.

DISCUSSION

Since the initial survey of crude extracts of 73 species of Caribbean sponges for their capacity to deter feeding of the generalist predatory fish *Thalassoma bifasciatum* in aquarium assays (Pawlik et al., 1995), bioassay-guided fractionation using aquarium and field assays has resulted in the isolation and identification of several metabolites responsible for sponge chemical defenses against fish predators: amphitoxin from *Amphimedon compressa* (Albrizio et al., 1995), oroidin and

TABLE 1. CONCENTRATION OF FRACTIONS FROM *Ircinia* spp. THAT DETERRED FISH FEEDING IN AQUARIUM AND FIELD ASSAYS[a]

Species	Concentration (mg/ml)		
	Fraction A	Fraction B	Total
Ircinia campana	4.17	3.08	7.52
Ircinia felix	5.23	3.84	9.07
Ircinia strobilina	3.35	2.43	5.78

[a] Fraction B was identified as the FTA variabilin **(1)**, while fraction A contains a mixture of the other FTAs.

related metabolites from *Agelas* spp. (Chanas et al., 1996; Assmann et al., 2000), stevensine from *Axinella corrugata* (Wilson et al., 1999), and triterpene glycosides from *Erylus formosus* (Kubanek et al., 2000). The present study confirms that the metabolites of *Ircinia* spp. that deter feeding by predatory fishes are the FTAs and not the foul-smelling volatile metabolites, as suggested by Zea et al. (1999) and Duque et al. (2001). Because the technique for transferring the volatile metabolites from macerated sponge tissue to the assay food did not rely on a precise quantitative method, but rather a comparison of the intensity of the odor, it is possible that the concentration of volatiles transferred to the assay food were below natural levels or that some oxidation of the volatiles had occurred in the process. However, it is just as likely that volatile concentrations exceeded natural levels in replicate experiments. In any case, we observed no hesitation on the part of the assay fish

TABLE 2. AQUARIUM ASSAY: CONSUMPTION OF FOOD PELLETS BY *Thalassoma bifasciatum* CONTAINING EXTRACTS AND ISOLATED METABOLITES FROM *Ircinia felix*[a]

Sample	Concentration (mg/ml)	Treated pellets eaten
Crude extract		0.33 (±0.33)
Crude extract minus fractions A + B		9
Fraction A	5.23 (natural)	0
	4.0	2
	2.0	4
	1.0	7
Fraction B	3.84 (natural)	0
	2.0	0
	1.0	2
	0.5	6
	0.25	9

[a] Fish consumed all 10 control pellets in each assay. $N = 10$ replicates for assays of the crude extract. For any individual assay, samples are considered deterrent if the number of pellets eaten is ≤ 6 ($P \leq 0.043$, Fisher exact test).

in consuming food pellets made from volatile-treated assay food in replicate experiments performed with two *Ircinia* spp., and these pellets retained the sponge odor throughout the assay process. We have used *Thalassoma bifasciatum* as an assay fish for testing the defensive properties of secondary metabolites from marine organisms for over 15 years (Pawlik et al., 1987) and have found that deterrent metabolites will elicit avoidance behavior in assay fishes (repetitive mouthing, opercular flaring, followed by subsequent consumption) at concentrations that are a fraction of those that result in immediate rejection. In this study, fish responses to volatile-treated pellets were no different from those to control pellets (immediate consumption). Given that these bad-smelling volatiles do not deter fish feeding in aquarium assays, it appears that they neither act as direct natural deterrents nor serve to signal the fish of the presence of FTAs in the sponge tissue (Duque et al., 2001).

Concentrations of FTAs were greatest in *I. felix*, followed by *I. campana* and *I. strobilina* (Table 1), as was also reported by Martínez et al. (1997) for the same species from the Colombian Caribbean. Levels of FTAs for all three species were several-fold higher than required to deter predation by generalist fishes (Table 2). Although concentrations of FTAs were lower in the surface tissues than in the sponge interior of *Ircinia* spp. (Zea et al., 1999), surface concentrations exceeded those necessary to deter fish predation, and it is likely that any bites taken by generalist predatory fishes would include both surface and internal sponge tissues.

Despite the presence of FTAs, *Ircinia* spp. are subject to predation by specialist sponge predators. In their survey of the diets of Caribbean fishes, Randall and Hartman (1968) reported that *I. strobilina* was found in the guts of five spongivorous species and was the most abundant sponge in the guts of three, comprising 30%, 9%, and 5.6% of the gut contents of the tilefishes *Cantherhines macrocerus* and *C. pullus* and the queen angel, *Holacanthus ciliaris*, respectively. *Ircinia* spp. were also consumed by some seastars (Wulff, 1995; Waddell and Pawlik, 2000a), although avoided by hermit crabs (Waddell and Pawlik, 2000b). Clearly, some fish and invertebrate predators have circumvented the chemical defenses of *Ircinia* spp.

Although other possible roles of the odor-producing volatile compounds from *Ircinia* spp. (e.g., antifouling, antiovergrowth) have not been studied and cannot be ruled out, it may be possible that these compounds are produced as metabolic by-products. For example, dimethylsulfide is an apparent waste product of the digestion of phytoplankton by zooplankton (e.g., Dacey and Wakeham, 1986). Similarly, volatile metabolites may be waste products of the digestion of phytoplankton (Pile, 1997) or of endosymbiotic bacteria (Simpson, 1984) by *Ircinia* spp. Nevertheless, the broad spectrum of bioactivities of known marine isocyanide and isothiocyanate compounds (Duque et al., 2001) and the high level at which they are produced and released from *Ircinia* spp. is certainly suggestive of a functional role for these metabolites.

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CHEMOSENSORY STIMULI FOR THE WALKING LEGS OF THE CRAYFISH *Procambarus clarkii*

FRANK S. COROTTO* and MICHELE R. O'BRIEN

Department of Biology
North Georgia College and State University
Dahlonega, Georgia 30597

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Abstract—Studies of chemoreception in crustaceans have shown that flesh-eating species can detect amino acids, nucleotides and derivatives, and amines, while most herbivorous and omnivorous species are additionally sensitive to carbohydrates. We used extracellular recording techniques to evaluate the effectiveness of a range of nitrogen-containing compounds (mostly amino acids), bile acids, and carbohydrates in stimulating chemoreceptor cells present in the second and third pereopods of the omnivorous crayfish *Procambarus clarkii*. When applied at a final concentration of $\sim$100 μM, effective stimuli were trehalose, leucine, cellobiose, glycine, sucrose, maltose, and ammonium (from most to least effective). The other 17 compounds tested, many chosen because they are potent stimuli for other crustaceans, were ineffective stimuli for *P. clarkii*. Concentration–response functions were determined for three single cells that were sensitive to ammonium, for five multiunit fibers sensitive to glycine, and for six multiunit fibers sensitive to leucine. Thresholds ranged from 10 nM to 10 μM, functions were generally linear when plotted against the log of the stimulus concentration, and there was little evidence of saturation. While *P. clarkii* is sensitive to only seven of 24 compounds tested, the compounds that proved stimulatory should serve as cues for location and identification of food items preferred by this omnivorous crustacean.

Key Words—Gustation, crayfish, chemoreception, taste, Crustacea, Procambarus.

* To whom correspondence should be addressed. E-mail: fcorotto@ngcsu.edu

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INTRODUCTION

Crustaceans use chemoreception to locate odor sources (Devine and Atema, 1982; Weissburg and Zimmer-Faust, 1993), discriminate between odors (Derby et al., 1989), and communicate with conspecifics (Atema, 1986). The range of stimulatory compounds varies among species, but typically includes amino acids, nucleotides and derivatives, amines and, in some cases, carbohydrates. Compounds that stimulate crustacean chemoreceptor cells are typically those that possess the best signal properties, i.e., they are found at high concentrations within food items, but are present at much lower "background" levels in the environment. For example, most amino acids are found in millimolar concentrations within the invertebrate tissues consumed by flesh-eating crustaceans, but in seawater are present in low nanomolar concentrations (Carr, 1988). Consequently, amino acids are recognized as some of the most important chemosensory stimuli for crustaceans, and sensitivity to amino acids has been demonstrated in a wide range of crustacean species.

In contrast, chemosensory sensitivity to carbohydrates has been demonstrated in just a few crustaceans, where such sensitivity appears correlated with herbivorous or omnivorous feeding habits. Carbohydrates are important chemical stimuli for the porcelain crab *Petrolisthes cinctipes* and fiddler crabs (*Uca* species), which feed largely on microscopic algae, for the omnivorous ghost crab *Ocypode quadrata* and the crayfish *Orconectes rusticus* (Hartman and Hartman, 1977; Robertson et al., 1981; Trott and Robertson, 1984; Tierney and Atema, 1988; Rittschof and Buswell, 1989). Carbohydrate sensitivity, however, is not found in all omnivorous crustaceans; the crayfish *Austropotamobius torrentium* is insensitive to sugars (Bauer and Hatt, 1980), and crayfish are widely considered to be omnivores (Lorman and Magnuson, 1978; Momot et al., 1978). More species must be studied to determine whether sensitivity to carbohydrates is a reliable predictor of herbivorous or omnivorous feeding habits in Crustacea.

One class of compounds that has not been tested on crustaceans is the bile acids. Bile acids are potent olfactory stimuli for fish (reviewed by Hara, 1994), which release bile acids in their feces (Zhang et al., 2001). If crayfish were able to sense bile acids, they might be able to identify fecal matter better upon encountering it. Moreover, zebrafish appear to respond to bile acids with the use of a different transduction mechanism and set of receptors than is used for other odorants (Michel and Derbidge, 1997; Michel, 1999). If crayfish chemoreceptor cells can also respond to bile acids, comparative studies of their transduction mechanisms could be performed.

Here we used physiological methods to investigate the range of chemical stimuli for the walking legs (pereopods) of the crayfish *Procambarus clarkii*. Crustaceans use their pereopods for locomotion and feeding, and for food localization (Devine and Atema, 1982; Steele et al., 1999) and evaluation (Hazlett, 1968). We

assessed the pereopod's sensitivity to a variety of nitrogen-containing compounds (mostly amino acids), carbohydrates, and bile acids.

METHODS AND MATERIALS

Animals and Solutions. Adult, intermolt *Procambarus clarkii* were obtained from a local supplier, maintained in aerated, aged tap water, and fed chicken, spinach, or carrots every other day. Second and third pereopods were excised at the middle of the merus to expose the nerve as it emerges from the proximal end of the carpus. Pereopods were placed in a conventional olfactometer that allowed superfusion of the dactyl and propus with a 20 ml/min carrier flow of modified van Harreveld's saline (205.0 mM NaCl, 13.5 mM $CaCl_2$, 5.4 mM KCl, 2.6 mM $MgCl_2$, and 2.4 mM HEPES), while the nerve was suspended in a separate compartment filled with the same solution. Pereopods were perfused with modified van Harreveld's saline (vH) via a cannula inserted into the articular membrane of the dactyl–propus joint. This perfusion technique, however, may not have efficiently provided vH to the most distal regions of the appendage. Poor perfusion in this area could compromise the passive flux of ions from the animal that is likely to provide the chemosensory dendrites with an environment protected from osmotic stress (see Discussion). By superfusing the entire appendage in vH, we hoped to prevent osmotic damage to these dendrites and to provide them with an environment similar to that normally generated by such a passive flux of ions.

Stimuli were injected by hand in 100 μl volumes into the vH carrier flow and reached peak concentration approximately 1 sec later, after being diluted $\sim$23-fold (Figure 1). Except in investigations of concentration–response characteristics, all stimuli were injected at a concentration of 2.3 mM to reach a predicted peak concentration of 100 μM at the pereopod.

Equipment. Exposed nerves were teased into fascicles, and activity was monitored with a conventional suction electrode connected to a DHS-300 active differential headstage (CB Sciences, Milford, Massachusetts, USA) and ETH-250 amplifier (CB Sciences) set to AC mode. Data were digitized at 10 kHz with the use of a PowerLab 200 A/D converter (ADInstruments, Milford, Massachusetts, USA) and monitored, stored, and analyzed on an Apple Macintosh computer equipped with Chart software (ADInstruments). Responses were quantified with the use of the MLS 062 Spike Histogram Extension (an optional component of Chart), which counted the total number of spikes with amplitudes falling within a certain range, set to exclude noise and the large action potentials generated by most mechanosensory axons.

Identification of Chemosensory Bundles. Axon bundles were considered chemosensory if they responded to either a fish food extract or a defined mixture—both were tested on every bundle. Fish food extract was prepared by grinding 5 g

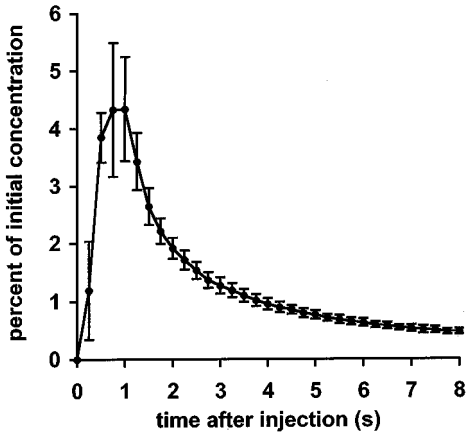


FIG. 1. Concentration profile of stimuli injected by hand into the olfactometer. Shown is the percent of the initial concentration of a 100 μl volume of 2 M NaCl injected into a carrier flow of 100 mM NaCl flowing at 20 ml/min, as determined by conductivity measurements. The stimulus concentration peaked within the olfactometer at 4.4% of the initial concentration. Mean values from 1–8 sec were fit best by the power function, $y = 4.12x^{-1.053}$ ($R^2 = 0.9988$; curve fitting was performed with Microsoft Excel). Based on this function, 60 sec after application of a stimulus, the stimulus concentration should be reduced to 1.3% of the peak concentration reached in the olfactometer. Bars show standard deviation, $N = 5$.

of Tetramin fish food (Tetra Sales, Blacksburg, Virginia, USA) in 100 ml of vH, mixing for 15 min, and filtering. Defined mixtures included all of the single compounds being tested, each at the same concentration as when they were applied individually.

Correction for Mechanosensory Activity. Most recordings included the activity of mechanoreceptor cells. When these cells generated large action potentials, their activity was excluded with the use of the MLS 062 Spike Histogram Extension. Remaining mechanoreceptor activity was excluded with two procedures. To eliminate activity induced by the carrier flow, the number of spikes occurring during the 5-sec period preceding the stimulus injection was subtracted from the number occurring during the 5-sec period following the injection. To eliminate responses elicited by the hand injection itself, responses to blank injections that occurred before and after injection of the stimulus were averaged and then subtracted from that elicited by the stimulus. Although necessary, this subtraction procedure likely led to small errors, mostly due to changes in the level of mechanoreceptor cell activity that tended to occur unpredictably as the procedure progressed. For example, if a non stimulatory compound is applied, but by chance evokes a greater mechanosensory response than the blanks, subtraction would yield a small, seemingly chemosensory response. Because of this problem, compounds

TABLE 1. COMPOUNDS INVESTIGATED IN INITIAL SURVEY AND TWO SUBSEQUENT INVESTIGATIONS

Initial survey	Second survey (11 bundles tested)
Nitrogen-containing compounds (18 bundles tested)	L-Arginine
Betaine	Glycine
L-Glutamate	L-Leucine
Glycine	L-Lysine
Ammonium chloride	*trans*-4-Hydroxy-L-proline
L-Serine	Mixture of all compounds used in the second survey
Taurine	Third survey (19 bundles tested)
Mixture of all N-containing compounds above	Cellobiose
Sugars (21 bundles tested)	β-Cyclodextrin
D-Glucose	D-Fructose
Lactose	D-Galactose
Maltose	D-Glyceraldehyde
Sucrose	Maltose
Mixture of all sugars above	D-Ribose
Bile acids (23 bundles tested)	Trehalose
Glycocholic acid	Mixture of all compounds used in the third survey
Taurocholic acid	
Taurodeoxycholic acid	
Mixture of all bile acids above	

were considered stimulatory only if they generated unambiguous responses upon visual inspection of the trace.

Compounds Tested and Protocols Used. We performed three surveys to explore the range of compounds that act as chemical stimuli for *Procambarus clarkii*. All chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The initial survey included nitrogen-containing compounds (mostly amino acids), sugars, bile acids, and mixtures of all the nitrogen-containing compounds, all the sugars, and all the bile acids (Table 1). Stimuli were applied in sets, e.g., all the sugars were tested, then all the bile acids, etc. The order of the sets was randomized as was the sequence of stimuli within each set. Stimulus blanks were applied between each set of stimuli to allow subtraction of mechanoreceptor cell responses. Fish food extract, or the defined mixture of all the single compounds, was also applied between each set of stimuli to assess viability. Data from a set of stimuli were excluded if the coefficient of variation of the two flanking viability checks exceeded 60%. This means that not all stimuli were tested on all axon bundles. For example, a bundle's responses to sugars would be excluded if they failed to meet the criterion for viability, but its responses to other sets of stimuli would still be included provided they met that criterion.

Initial results indicated that glycine was a potent stimulus. In the lobster *Homarus americanus*, glycine-sensitive cells are also often sensitive to hydroxyproline, arginine, lysine, and leucine (Tierney et al., 1988; Corotto et al., 1992;

Voigt and Atema, 1992). To determine if this might also be the case in *Procambarus clarkii*, we conducted a second survey that included those stimuli (Table 1). Early results also indicated that maltose was a potent stimulus. Our third survey included a range of carbohydrates to explore carbohydrate sensitivity further (Table 1). In the second and third surveys, data were again excluded if the coefficient of variation for the two flanking viability checks exceeded 60%.

By fitting the stimulus profile (Figure 1) to a power function, we determined that allowing 1 min between stimulus injections was sufficient to remove more than 98% of the injected stimulus. We, therefore, allowed 1 min to pass between stimulus applications. Any systematic effects from the remaining stimulus were eliminated by randomizing the order of stimulus presentation.

Quantification of Results. When analyzing multiunit recordings, investigators commonly express responses relative to that elicited by a standard stimulus, tested on all fibers. We chose not to employ this method because it results in all axon bundles being given equal weight regardless of the number of chemosensory axons they contain; fascicles containing few chemosensory axons, thus displaying low levels of activity, would influence the results just as much as fibers containing many chemosensory axons, which display greater levels of activity. Rather than expressing responses relative to that elicited by a standard stimulus, we chose to calculate the average number of action potentials elicited by a stimulus per bundle tested. This approach allows larger fascicles, with more chemosensory axons, to have a proportionally greater influence on our results than that of smaller, less representative fibers.

Concentration–Response Determinations. To determine concentration–response characteristics, we applied glycine, leucine, or ammonium at a predicted peak concentration of 100 μM to test for chemical sensitivity. We initially chose ammonium because we found that identifiable single cells were typically ammonium-sensitive. However, due to the rarity with which we encountered single units (see Results), we chose to evaluate the concentration–response characteristics of glycine and leucine with the use of multiunit recordings. After locating a chemically sensitive fiber, we allowed 5 min to pass and then applied an ascending concentration series, in log unit steps, beginning with a predicted, final concentration of 1 nM and ending with 1 mM.

Preliminary results of this study have been published in abstract form (Corotto and Gentilozzi, 2000).

RESULTS

Fifty-six nerve bundles displaying chemosensory activity were identified in this study. Fascicles typically responded to chemical stimuli with large numbers of action potentials from many different cells (Figure 2). Chemosensory spike

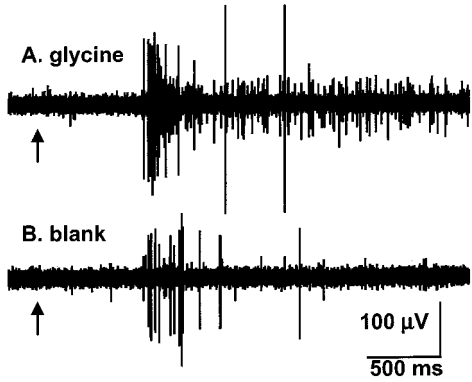


FIG. 2. Typical suction-electrode recordings obtained from nerves in the pereopods of *Procambarus clarkii*. (A) Injection of glycine at a predicted peak concentration of 100 μM elicited large amplitude action potentials from mechanosensory neurons and a sustained train of smaller spikes from many chemosensory cells. (B) Injection of a blank elicited only the mechanosensory response. Arrows indicate time of injection.

amplitudes ranged from being barely visible to $\sim$100 μV. Multiunit chemosensory responses were typically superimposed upon those of numerous mechanosensory axons, many of which possessed spike amplitudes of 100 μV or more. Of the 56 chemosensory bundles we studied, only seven appeared to display the activity of single chemosensory cells.

The most stimulatory single compound tested was the disaccharide trehalose (Figure 3). This is followed, in order of decreasing effectiveness, by leucine, cellobiose, glycine, sucrose, maltose, and ammonium. Unambiguous responses were not obtained from the other 17 single compounds tested (shown in Table 1, but omitted from Figure 3). A single, unambiguous response to the equimolar mixture of bile acids was obtained once (not shown), but single bile acids failed to evoke convincing responses from that fiber.

Of the seven single cells identified, six responded to ammonium and one to glycine. Thus, ammonium sensitivity dominated the small number of cells identifiable as single units, even though ammonium was the weakest chemosensory stimulus identified, eliciting the lowest average number of spikes per bundle (Figure 3).

Concentration–response functions from multiunit and single-unit recordings showed thresholds at predicted, final stimulus concentrations of 10 nM to 10 μM (Figure 4). Two of the three single-units displayed concentration–response functions that appeared roughly linear when plotted against the log of the stimulus concentration (Figure 4A). The third single unit generated fewer spikes to 1 nM ammonium than to 100 μM ammonium, indicating saturation. Multiunit responses

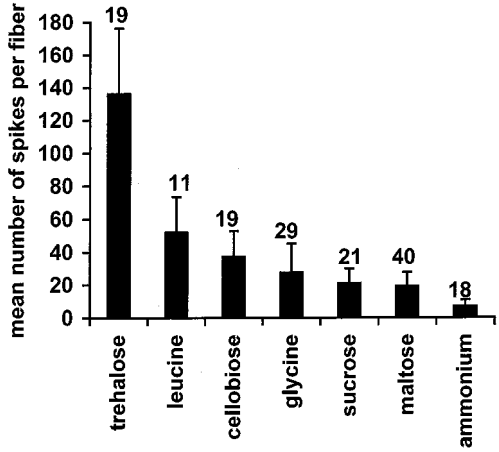


FIG. 3. Mean number of spikes per bundle elicited by chemical stimuli applied at a predicted peak concentration of 100 μM. Numbers above error bars indicate the number of axon bundles tested. In a few cases, both recognizable single cells and multiunit activity was present in the same recordings. These were analyzed separately and considered separate bundles. Bars show standard error of the mean.

to leucine and glycine generated concentration–response functions that showed little sign of saturation and were also approximately linear in form (Figure 4B).

DISCUSSION

The crayfish *Procambarus clarkii* proved a challenging subject for an electrophysiological investigation of chemoreception. Suction electrode recordings from axon bundles usually displayed the activities of large numbers of mechanoreceptor cells with only a small minority including chemoreceptor cell responses. When present, chemosensory responses typically consisted of the activities of large numbers of cells (Figure 2). In contrast, recordings obtained with similar techniques from the maxillipeds of the lobster *Homarus americanus* contain relatively few mechanoreceptor cell responses, abundant chemoreceptor cell responses, and the activities of single chemoreceptor cells are often identifiable (personal observations). While we had initially hoped to focus on the responses of single units, one advantage of multiunit recordings is that they allow one to study a vast number of cells, although it is impossible to determine the actual number. We identified seven single cells, and six of them were selective for ammonium, despite the fact that ammonium is the least effective of the compounds we identify as being stimulatory (Figure 3). This suggests that cells sensitive to ammonium are more easily identified as single units than cells sensitive to other

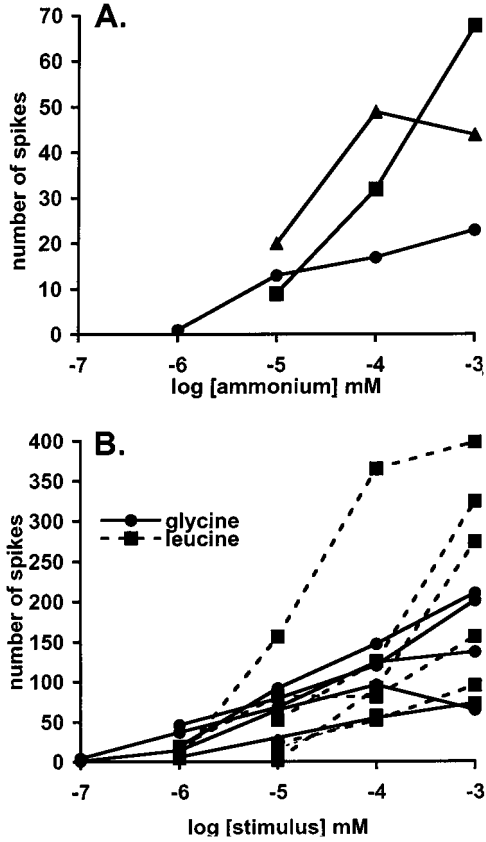


FIG. 4. Concentration-response functions of chemoreceptor cells from *P. clarkii*. (A) Responses of three single cells to different predicted peak concentrations of ammonium. (B) Responses of multiunit fibers to different predicted peak concentrations of glycine and leucine.

compounds. Perhaps ammonium-sensitive cells have larger axons, and, therefore, generate larger spikes in extracellular recordings, or perhaps they are bundled differently within the nerve. Regardless, the observation that most single cells were sensitive to a weak stimulus suggests that focusing entirely upon the responses of single cells may bias one's results towards stimuli that affect particular classes of cells most likely to be identified as single units in extracellular recordings.

Even though many of the compounds we tested are potent stimuli for other crustaceans, only seven evoked unambiguous physiological responses from the pereopods of *P. clarkii*. Neurophysiological and behavioral studies have shown

that sensitivity to taurine, betaine, hydroxyproline, serine, and especially glutamic acid is widespread in crustaceans (Levandowsky and Hodgson, 1965; Fuzessery and Childress, 1975; Robertson et al., 1981; Derby and Atema, 1982; Hatt, 1984; Laverack, 1988; Tierney and Atema, 1988; Voigt and Atema, 1992). None of these compounds is an effective physiological stimulus for the pereopods of *P. clarkii*. We cannot, however, exclude the possibility that behavioral studies, or neurophysiological studies of other appendages, might reveal sensitivity to these compounds. The stimulatory compounds we did identify seem, nevertheless, highly appropriate as cues for food localization and identification. Crayfish are omnivores (Lorman and Magnuson, 1978; Momot et al., 1978) with *P. clarkii* subsisting mostly on living plants and plant detritus along with smaller quantities of invertebrates (Penn, 1943; Ameyaw-Akumfi, 1977; Avault, et al., 1983; Feminella and Resh, 1989). It is within the body fluids of these invertebrates that *P. clarkii* is most likely to encounter the disaccharide trehalose (Fairbairn, 1958), the most stimulatory compound we identified. Other effective carbohydrates include cellobiose, maltose, and sucrose. Sensitivity to cellobiose and maltose is appropriate for an animal that feeds on plant detritus; these disaccharides are produced by the microbial degradation of cellulose and starch (Ljungdahl and Eriksson, 1985). Sucrose, another effective disaccharide, is the major sugar found in phloem sap (Mohr and Schopfer, 1995) and would be present in the fresh macrophytes that *P. clarkii* consumes. Other effective stimuli for *P. clarkii* include leucine, glycine, and ammonium. Leucine and glycine are common amino acids, and glycine, in particular, is found at high concentrations in the tissues of many invertebrates (Simpson et al., 1959; Awapara, 1962). Ammonium is a common metabolite and is also a potent stimulus for many crustaceans. Collectively, the stimulatory compounds we identified should render the animal highly sensitive to its natural food items, including invertebrates, plant detritus, and fresh macrophytes.

The particular compounds we identified as chemosensory stimuli for *P. clarkii* closely match those shown to stimulate feeding behavior in the crayfish *Orconectes rusticus*. In *O. rusticus*, the most powerful stimulants of feeding behavior are cellobiose, sucrose, glycine, and maltose (Tierney and Atema, 1988). All of these are effective physiological stimuli for *P. clarkii*. Similarly, monosaccharides are ineffective for both species. In contrast, physiological investigations of pereopods from the crayfish *Austropotamobius torrentium* show that this animal responds to a very different range of compounds. *A. torrentium* possesses three classes of chemoreceptor cells: one tuned to amino acids, one to amines, and one to pyridines (Bauer and Hatt, 1980). The most effective amino acid stimulant for *A. torrentium* is serine (Bauer et al., 1981; Hatt, 1984), an ineffective stimulus for *P. clarkii*. Sugars are ineffective stimuli for *A. torrentium* (Bauer and Hatt, 1980), but for *P. clarkii* some of the most potent stimuli are disaccharides. Additional studies on *P. clarkii* are necessary to assess its sensitivity to amines and pyridines, but even without testing those classes of compounds, it is clear that the pereopods

of the two species are sensitive to a different range of chemical stimuli. It would be interesting to know if these chemosensory differences are also paralleled by differences in dietary preference.

To our knowledge, this is the first investigation of bile acids as potential chemosensory stimuli for a crustacean; bile acids are especially potent odorants for fish (reviewed by Hara, 1994). Crayfish would likely encounter bile acids in fish feces (Zhang et al., 2001). We had thought that sensitivity to bile acids might aid crayfish in identifying fecal matter and, presumably, allow them to reject it as food. The three bile acids we chose to investigate, however, were not effective chemosensory stimuli for *P. clarkii*. We observed one unambiguous response to the mixture of all three bile acids but did not observe responses to the components. Since only three bile acids were included in this study, investigations with others are necessary before we can conclude that they are ineffective, in general, as chemosensory stimuli for *P. clarkii*.

In crustacean chemoreceptor cells, physiological thresholds for the most potent stimuli typically range from 1 to 100 nM (Fuzessery and Childress, 1975; Derby and Atema, 1982; Tierney et al., 1988; Corotto et al., 1992). Although values higher and lower than these have been noted (Laverack, 1963; Levandowsky and Hodgson, 1965; Fuzessery et al., 1978; Derby and Atema, 1982), unusually high thresholds may result from testing compounds that are not a cell's best stimulus. This is less of a problem with multiunit recordings because the compound being applied is likely to be the best stimulus for at least some of the cells being tested. Our findings indicate that *P. clarkii*'s thresholds may be slightly higher than the typical 1–100 nM range; two multiunit fibers responded to 100 nM glycine but not 10 nM glycine, and the other multiunit fibers had higher thresholds (Figure 4B). The three single cells we identified also had thresholds above 100 nM (Figure 4A). Exact concentrations, however, are difficult to predict when using conductivity measurements to determine the concentration of a diluted stimulus, as we did. Such measurements provide an estimate of the average concentration surrounding the pereopod, but turbulence within the olfactometer is likely to have distributed the stimulus in the form of small patches of higher and lower concentrations, so the actual concentration of a stimulus at a particular chemoreceptor cell may differ from that predicted. Nevertheless, our results are sufficient to conclude that the thresholds of chemoreceptor cells of *P. clarkii* are similar to, or perhaps slightly higher than those of most other crustaceans. Interestingly, chemoreceptor cells from the blue crab *Callinectes sapidus* lose some sensitivity as the animal moves from seawater to a freshwater environment (Gleeson et al., 1997). Apparently the accompanying osmotic stress causes shortening of the exposed chemosensory dendrites (Gleeson et al., 1996). Chemosensory dendrites in *P. clarkii* are presumably exposed as well, so we had thought that *P. clarkii*'s chemoreceptor cells might be forced to operate at unusually high thresholds given its freshwater environment. This is not the case. Similarly, response thresholds

of amino acid-sensitive cells in the crayfish *Austropotamobius torrentium* lie at approximately 300 nM for serine, the most potent amino acid stimulus (Bauer et al., 1981). In *Callinectes sapidus*, passive flux of ions from the animal provides a microenvironment surrounding the chemosensory dendrites that prevents complete osmotic lysis and allows chemoreception to occur, although responses are reduced in magnitude (Gleeson et al., 1997, 2000). A similar microenvironment may protect chemosensory dendrites in crayfish.

Of the seven compounds identified as stimulatory for *P. clarkii*, the most effective was the disaccharide trehalose. Trehalose elicited more than twice the average number of spikes per bundle as leucine, the next most effective stimulus (Figure 3). Trehalose sensitivity has been noted previously in the porcelain crab *Petrolisthes cinctipes* (Hartman and Hartman, 1977), the fiddler crabs *Uca pugilator* and *U. longisignalis* (Robertson et al., 1981; Weissburg and Zimmer-Faust, 1991), and the ghost crab *Ocypode quadrata* (Trott and Robertson, 1984). Because trehalose is such a potent stimulus for *P. clarkii*, we wonder if it might play a unique role as a chemical cue. Could invertebrate prey release enough trehalose by diffusion across their gills or body wall for crayfish to detect it at a distance? If not, what if the prey item were wounded? Crayfish, including *P. clarkii*, use chemosensory cues to locate distant odor sources (Kreider and Watts, 1998; Moore and Grills, 1999; Steele et al., 1999), and trehalose would seem to be an ideal chemical signal for indicating the presence of a nearby protein-rich meal in the form of a wounded invertebrate.

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ANTAGONISM BETWEEN JASMONATE- AND SALICYLATE-MEDIATED INDUCED PLANT RESISTANCE: EFFECTS OF CONCENTRATION AND TIMING OF ELICITATION ON DEFENSE-RELATED PROTEINS, HERBIVORE, AND PATHOGEN PERFORMANCE IN TOMATO

JENNIFER S. THALER,[1,*] ANA L. FIDANTSEF,[2,3] and RICHARD M. BOSTOCK[2]

[1]*Department of Botany, 25 Willcocks Street*
University of Toronto
Toronto, Ontario M5S 3B2 Canada

[2]*Department of Plant Pathology*
University of California
Davis, California 95616

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Abstract—The jasmonate (JA) and salicylate (SA) signaling pathways in plants provide resistance to herbivorous insects and pathogens. It is known that these pathways interact, sometimes resulting in antagonism between the pathways. We tested how the timing and concentration of elicitation of each pathway influenced the interaction between the jasmonate and salicylate pathways measured in terms of five biochemical responses and biological resistance to caterpillars and bacteria. The salicylate pathway had a stronger effect on the jasmonate pathway than did the reverse. The negative signal interaction was generated by two distinct paths in the plant. A negative interaction in the biochemical expression of the two pathways was most consistent in the simultaneous elicitation experiments compared to when the elicitors were temporally separated by two days. Herbivore bioassays with *Spodoptera exigua* also consistently reflected an interaction between the two pathways in the simultaneous elicitation experiments. The negative signal interaction reducing biological resistance to the herbivore was also demonstrated in some temporally separated treatment combinations where attenuation of the biochemical response was not evident. Concentration of the elicitors had an effect on the pathway interaction with consistent biochemical and biological antagonism in the high concentration experiments and inconsistent antagonism in the low concentration experiments.

* To whom correspondence should be addressed. E-mail: thaler@botany.utoronto.ca
[3] Current address: Novozymes Biotech, Inc. 1445 Drew Avenue, Davis, California 95616.

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The bacterial pathogen, *Pseudomonas syringae* pv. *tomato* (*Pst*), consistently showed reduced lesion development on plants with SA responses activated and, in some experiments, on JA-elicited plants. Resistance to *Pst* was not reduced or enhanced in dual-elicited plants. Thus, signal interaction is most consistent when elicitors are applied at the same time or when applied at high doses. Signal interaction affected the herbivore *S. exigua*, but not the pathogen *Pst*.

Key Words—Signal interaction, induced defense, jasmonate, salicylate, *Spodoptera exigua*, *Pseudomonas syringae* pv. *tomato*, polyphenol oxidase, tomato.

INTRODUCTION

Plants can be attacked by many organisms at a particular moment and over their lifetimes. They respond to this attack by inducing responses that can protect themselves against future herbivory. Two suites of responses that are important in plant resistance have jasmonic acid (JA) and salicylic acid (SA) as key signal components. Jasmonate-mediated responses and salicylate-mediated responses (also known as systemic acquired resistance, SAR) are necessary for plant resistance against insects and pathogens, respectively. Biochemical interactions in the expression of the JA and SA pathways can prevent the maximal simultaneous expression of each pathway (Felton and Korth, 2000). This has led researchers to ask under what conditions these signal interactions occur, as this will affect their ecological consequences. The biochemical interactions that occur to generate negative signal interactions may be influenced by the temporal pattern of induction and degree of elicitation (Doherty et al., 1988).

There are several ways in which the jasmonate and salicylate pathways are known to interact. SA itself interferes with JA signaling in at least three sites. The first site is before JA synthesis where 13*S*-hydroperoxylinolenic acid is converted to 12-oxy-phytodienoic acid by 13*S*-hydroperoxide dehydrase (Peña-Cortez et al., 1993; Doares et al., 1995a), the second is in the conversion of 12-oxy-phytodienoic acid to JA (Engelberth et al., 2001), and the third is after JA synthesis (Doares et al., 1995a). Compounds structurally similar to SA have also been shown to inhibit the JA pathway (Doherty et al., 1988). For example, acibenzolar-*s*-methyl (benzothiadiazole, BTH), a synthetic SAR inducer, is translocated in the plant and can, therefore, activate resistance systemically. Thus, while application of BTH does not result in increased endogenous SA, BTH apparently shares sufficient structural similarity to SA to have the same functional properties in vivo. The inhibitory effects of JA on the SA pathway are less well documented (but see Niki et al., 1998; Schenk et al., 2000). JA may interfere with salicylate biosynthesis in tobacco plants via a G-protein switch that is required for a plant's appropriate response to external stimuli (Seo et al., 1995).

The signal molecules (e.g., JA and SA) that initiate the plant responses and appear to be involved in generating the pathway interactions (Felton et al., 1999)

decay with time after elicitation (Niki et al., 1998). Jasmonate levels increased in mechanically wounded leaves of tomato plants sixfold within 90 min and then dropped over the next several hours (Doares et al., 1995b; Conconi et al., 1996). Salicylic acid levels increase in *Pseudomonas syringae* pv. *tomato* (*Pst*) -infected tomato plants and remained twofold higher for at least 48 hr (Oldroyd and Staskawicz, 1998). This suggests that the signaling interaction between the JA and SA pathways will be influenced by the timing of elicitation and that SA induction will influence JA induction more than the converse (Creelman and Mullet, 1997). However, these endogenous JA and SA measures are not directly comparable because the mechanical damage was a single pulse of stress and *Pst* infection was chronic. In addition, other components of the jasmonate and salicylate signal pathways may contribute to the signal interaction and be differentially responsive to the timing of elicitation (Felton and Korth, 2000).

Plants respond to natural damage and induction by elicitors in a dose-dependent manner (Karban and English-Loeb, 1988; Enyedi et al., 1992) and the jasmonate/salicylate signal interaction may be influenced by the degree to which each individual pathway is induced. It may be that heavy infection by a plant pathogen influences deployment of resistance to insects more than a mild infection. For example, Niki et al. (1998) examined antagonisms in expression of JA- and SA-inducible mRNA and proteins when tobacco leaf disks were floated in solutions containing the two elicitors. They found that the concentration of each elicitor negatively influenced the action of the other in a dose-dependent manner. Doherty et al. (1988) found an effect of timing in salicylic acid application on proteinase inhibitor production. Brief application of aspirin to cut tomato stems inhibited production of proteinase inhibitors by mechanical wounding, but only for 2-3 hr after the aspirin application. Doares et al. (1995a) found that aspirin inhibited systemin (a mobile systemic signal of octadecanoid induction in tomato plants) when applied either at high concentration (10 mM) for a short duration (30 min) or low concentration (1 mM) for a long duration (24 hr).

Given this antagonistic interaction in the expression of resistance, we studied how elicitor concentration and timing of application affect the biochemical expression of each pathway and resistance to insects and pathogens. To do this we first needed to (1) establish a concentration response and time course for the effects of BTH on tomato plants [JA concentration response–time course reported in Thaler et al. (1996)] and (2) test whether the interactions between the JA and SA pathways were due to the washing off of elicitor in the dual spray treatments. These preliminary experiments established the range of concentration and timing conditions where pathway interactions could occur and showed that pathway interactions were not result of interactions between elicitors at the leaf surface. (3) Based on these results, we determined how concentration and timing of elicitation influenced the interaction between the JA and SA pathways. Our hypothesis was that the negative interactions between the JA and SA pathways would be

weaker or not occur at lower concentrations of elicitor or when elicitation was temporally separated. (4) Lastly, we examined the effect of BTH on *Spodoptera exigua* caterpillars feeding on wild-type plants and jasmonate-deficient plants. The last experiment was conducted to demonstrate conclusively that the positive effect of BTH treatment on caterpillar growth (observed in 1 and 3 above) was indeed due to an effect of BTH on the jasmonate pathway. If so, the positive effect of BTH on herbivore performance seen in wild-type plants should not be detected in the jasmonate deficient plants.

We assessed this interaction between pathways by assaying plant defensive compounds: PPO (polyphenol oxidase), POD (peroxidase), and LOX (lipoxygenase) enzyme activities, and P4 (pathogenesis-related protein 4) and PINII (proteinase inhibitor) mRNA accumulation, as well as herbivore and pathogen performance: relative growth rate, gross growth efficiency, and leaf area consumed by *S. exigua* caterpillars, and lesion formation caused by *Pst*.

METHODS AND MATERIALS

General Protocols

Tomato plants (*Lycopersicon esculentum* cv. New Yorker) were grown in 4-in. pots containing UC soil mix in a greenhouse. Plants were grown until the four-leaf stage, after which the treatments were applied. JA and BTH were applied to plants using hand-held atomizers. The JA was prepared in aqueous suspension using acetone to help disperse it in water (1% acetone in water). BTH was dissolved in water. The solutions/suspensions were sprayed to runoff onto the desired portion of the plant, shielding the rest of the leaves. Control plants were sprayed with an equal amount of water with acetone.

Two days after the plants were treated, we measured the levels of hallmark indicators of each pathway to determine the level of activation of the SA- and JA-mediated responses. We measured P4 mRNA as a marker of BTH action, and PPO activity and PINII mRNA as markers for JA action. POD and LOX activity, other enzymes with defense-related activity, were also measured. The fourth youngest leaf (systemic, unelicited) was used in most cases, although the third leaf (local, elicited) was used for some experiments as noted in the figures. Thus, we tested for systemic induction unless stated. The terminal leaflet was collected for PPO, POD, and LOX assays unless *Pst* was inoculated on this leaflet, in which case one of the adjacent leaflets was used for biochemical analyses. The other adjacent leaflet was collected for the herbivore bioassay. The remainder of the leaf from four plants were collected and pooled for each P4 and PINII mRNA sample.

RNA Gel Blot Analyses. Abundance of P4 and PINII mRNA was determined by analysis of total RNA (20 μg) prepared from tomato leaves (Choi et al., 1994) according to the methods in Fidantsef et al. (1999). When both *pinII* and

*p*4 expression were measured, the blots were hybridized with the *pinII* probe and then stripped and hybridized with the *p*4 probe. Quantitation of hybridization was measured by exposing the probed blots to a screen that was scanned using a phosphor imaging system (Storm 860 scan, Molecular Dynamics, Inc., Sunnyvale, California, USA). The intensity of the bands produced was measured by drawing a box around the bands and measuring the counts in the area. Equal loading of RNA samples was verified by hybridization of the blots with a tomato cyclophilin DNA probe or by staining of rRNAs with ethidium bromide.

Enzyme Activity. PPO and POD activities were determined according to the methods described in Thaler et al. (1996). Briefly, we extracted the enzymes from weighed leaflets that were homogenized in ice-cold buffer and then centrifuged to obtain a clarified extract for enzyme analyses. For PPO the supernatant was added to a caffeic acid solution (2.92 mM in pH 8 K Phos buffer); for POD the supernatant was added to a guaiacol solution (2.92 mM in pH 8 K Phos buffer), and for LOX the supernatant was added to a linoleic acid suspension (0.4 mM linoleic acid in 0.1 M, pH 8, K Phos buffer with 0.1% Tween 20).

Herbivore Bioassays. Newly hatched *S. exigua* caterpillars were reared on artificial diet (Southland Products) for three to five days before the bioassays. Caterpillars were placed on a single leaflet in 90-mm Petri dishes lined with moist filter paper. The caterpillars were weighed at the beginning of the experiment and again at the end, after two to three days of feeding on the specified leaflets. The assays were terminated before the caterpillars could consume the entire leaflet. Relative growth rate (RGR) was calculated [(final weight-initial weight)/(initial weight $\times$ number of days)] (Waldbauer, 1968). The amount of leaf area consumed (AREA) was quantified using an acetate grid and gross growth efficiency (ECI) was calculated as [(final weight – initial weight)/area consumed)].

Bacterial Speck Disease Assay. Pseudomonas syringae pv. *tomato* isolated from field-grown tomatoes (isolate Pst23, gift of D. Cooksey, Department of Plant Pathology, UC Riverside) was incubated at 27°C for 48–72 hr on King's B medium, and colonies were suspended in sterile water. An aqueous suspension of 10^7 colony-forming units (CFU)/ml was gently painted on the terminal leaflet of the fourth leaf of intact plants with a cotton applicator, and the plants were incubated in the greenhouse. Seven days later, the number of lesions on the inoculated leaflet were counted. There is a strong correlation between the number and/or amount of lesions and bacterial populations (colony-forming units per square centimeter) (Craig Richael, personal communication).

Experimental Protocols

Concentration Response. Concentration response curves for the effects of JA and BTH in tomato were established. The effect of applications of four concentrations of JA on several defensive proteins was reported in Thaler et al. (1996).

BTH concentration response data are reported here. To standardize the treatment, we applied BTH to the third leaf (all leaflets) only. We measured P4 mRNA levels (total density, counts), PPO (ΔOD per gram per minute), POD (ΔOD per gram per minute) activity in the third leaf, and 24-hr RGR, AREA (square millimeters of leaf area consumed), and ECI of *Trichoplusia ni* (a generalist noctuid caterpillar) at 0, 0.3, 0.6, 1.2, and 2.5 mM BTH applications. The terminal leaflet was used for PPO and POD quantification, and the adjacent leaflet was used for the herbivore bioassay. Ten replicate plants were used for each of the five concentration treatments. P4 expression was also quantified at 0.0012, 0.012, 0.036, and 0.12 mM BTH application to establish the minimum concentration required for induction. In addition, we assayed *Pst* lesion formation at three concentrations (0, 0.3, and 1.2 mM) two days after plants were elicited ($N = 11$ plants per concentration). *Pst* was inoculated on the terminal and adjacent leaflets of the elicited leaf two days after BTH elicitation, and lesions were counted seven days later. We used linear regression to analyze the effect of BTH concentration on plant responses, insect performance, and lesions. Mortality of caterpillars slightly reduced sample sizes for RGR, AREA, and ECI.

Because we frequently found that plants sprayed with BTH supported relatively high growth of herbivores (see results), we tested for the effects of BTH on total leaf protein content in the elicited leaf. Total protein was assayed using the Bradford method as modified for plant protein by Jones et al. (1989). Ten replicates of each treatment were analyzed in the control, 0.3, 0.6, 1.2, and 2.5 mM BTH treatments. We used linear regression to analyze the effect of BTH application on plant protein content.

Time Course. To establish the timing of induction, we determined the effects of 1.2 mM BTH on local P4 mRNA induction 1, 4, and 8 days after treatment. The third leaf was elicited with BTH and extracted for P4 mRNA. One sample of each treatment was analyzed, with each sample consisting of elicited leaves pooled from four plants. We used linear regression to analyze the effect of BTH application on P4 mRNA accumulation.

Elicitor Runoff Experiment. Spraying plants with both elicitors simultaneously could possibly wash some elicitor off the plant and produce the antagonism that we observed. To evaluate this possibility, we simultaneously sprayed plants with each elicitor and water and compared the responses to each elicitor alone. The third leaf was sprayed with 1.5 mM JA alone, JA and water, 1.2 mM BTH alone, or BTH and water ($N = 8$ each treatment). The terminal leaflet of the third leaf was assayed for activity of PPO, and the rest of the leaf was collected for P4 mRNA accumulation analysis. Leaves from two plants were pooled for each P4 sample ($N = 3$ each treatment). This experiment was conducted concurrently with a trial where JA induced PPO, BTH induced P4 mRNA, and there was a significant reduction in PPO activity and P4 transcript accumulation when plants

were dually elicited compared to single elicitation with JA or BTH, respectively (data not shown). We used *t* tests to compare PPO activity in JA and JA + water treatments, and P4 mRNA abundance in BTH and BTH + water treatments.

Pathway Interaction Experiments. To examine the effect of the concentration and timing of elicitation on the antagonistic interactions between JA- and SA-mediated resistance, we conducted a series of experiments where we employed four general treatments: plants elicited with (1) water (control), (2) JA, (3) BTH, and (4) JA and BTH.

Simultaneous Elicitation. The third leaf of plants at the four-leaf stage was sprayed with the elicitors. Two concentrations of JA and BTH were used: low JA concentration = 0.5 mM (approx. 0.105 mg/plant) and high JA concentration = 1.5 mM (approx. 0.315 mg/plant) and low BTH concentration = 0.3 mM (approx. 1.0 μmol/plant) and high BTH concentration = 1.2 mM (approx. 3.0 μmol/plant). The two experimental combinations of our four treatments were: (1) high JA, high BTH, simultaneously (three trials) and (2) low JA, low BTH, simultaneously (4 trials). The low and high concentrations were chosen based on the concentration–response curves generated above. The low concentration treatment of our elicitors resulted in a full chemical response (i.e., without excess elicitor), while the higher concentration resulted in excess elicitor in the plant (i.e., more elicitor than necessary to get the maximum response).

Delayed Elicitation. In addition, two timing regimes were used to test the hypothesis that the negative interactions between the JA and SA pathways would not occur when elicitation of the respective pathways was temporally separated. These experiments consisted of treating plants first with jasmonate followed by BTH or first with BTH followed by jasmonate, and were conducted using the high and low concentrations of JA and BTH. The four experimental combinations of our four treatments were: (3) high JA, two-day delay, high BTH (1 trial); (4) high BTH, two-day delay, high JA (2 trials); (5) low JA, two-day delay, low BTH (2 trials); and (6) low BTH, two-day delay, low JA (2 trials). In these experiments, we assayed PPO, POD, and LOX enzyme activities, P4 and PINII mRNA accumulation, relative growth rate, leaf area consumed, and conversion efficiency of *S. exigua* caterpillars, and lesions formed in response to*Pst*. Sample sizes for each response variable are given in the figures. Not all chemical and biological assays were conducted during every trial; the specific information for each trial is presented in the figures and tables.

Factorial analysis of variance was used to analyze the effects of elicitor treatments on the respective response variables. The main effects are interpreted in a manner similar to a single-factor analysis if the JA × BTH interaction is not significant. In this case, the main effects indicate whether JA and BTH affect the response variable when applied alone, and these effects are simply additive in the dual-elicitation treatment. If the interaction is significant, then the main effects

are not independent and cannot be interpreted individually (Underwood, 1997). A significant interaction means that the effects of JA and BTH were not additive, i.e., the effect in the dual elicitation treatment (relative to control) is not the sum of the individually elicited treatments. Thus, there are two possible scenarios in which the two plant defense pathways negatively interact. The first is when both main effects are significant but operate in the opposite direction (e.g., JA decreases RGR, BTH increases RGR) and the interaction term is not significant (i.e., effects of JA and BTH are additive); the net result is an intermediate response in the dual elicited plants. The second scenario is a statistically significant interaction indicating that the expression of one response pathway influenced the expression of the other. In this case, inspection of the means indicates the nature of the interaction in dual-elicited plants compared to singly elicited plants. For example, if JA singly increases PPO, BTH singly has no effect, but together JA and BTH result in lower PPO activity than JA alone, then we expect a significant statistical interaction between elicitation by JA and BTH. Both of these scenarios are biologically relevant (see Discussion).

Data were log $x+1$-transformed where necessary to equalize variance and meet the assumptions of ANOVA. Untransformed means are presented in graphs. Although "trial" was always included in the analyses, trials of the same experimental treatments were pooled for graphical presentation because results across trials were consistent. Only one replicate of PINII mRNA per treatment was measured in each experiment and, therefore, was not statistically analyzed.

S. exigua Performance on Wild-Type and Jasmonate-Deficient Plants. If BTH benefits herbivores through its suppression of the jasmonate pathway, then herbivores should have increased performance on wild-type plants elicited with BTH, but not on jasmonate-deficient plants elicited with BTH. We tested this by examining herbivore performance on the jasmonate-deficient mutant defenseless-1 compared to its wild-type counterpart Castlemart (Howe et al., 1996). This mutant is deficient in its ability to synthesize jasmonic acid. Constitutive PPO activity is not different between the wild-type and jasmonate-deficient plants. PPO activity is induced in wild-type plants when they are damaged by *S. exigua* caterpillars, but not in jasmonate-deficient plants that are damaged by caterpillars (J. S. Thaler and M. Dicke, unpublished data). Wild-type and jasmonate-deficient plants were divided into two groups; one group was elicited with 1.2 BTH on the third leaf, and the other group was treated with the BTH carrier as the control. After three days, the terminal leaflet of the third leaf was excised, placed in a damp Petri dish, and one *S. exigua* neonate was added. This neonate was allowed to develop for three days, after which survivorship was scored and mass measured. This experiment was repeated three times with 12–20 plants per treatment per trial. Survivorship was pooled across trials and analyzed using a G test. Mass was analyzed using a two-way ANOVA for each plant type with BTH treatment and trial as the factors.

RESULTS

BTH Concentration Response. Tomato plants responded to BTH by inducing P4 at concentrations as low as 0.0012 mM, but the effect was 20-fold at 0.6 mM and 40-fold at 1.2 mM (regression: $R^2 = 0.737$, $F_7 = 19.631$, $P = 0.003$; Figure 1). POD was induced by BTH in a concentration-dependent manner ($R^2 = 0.299$, $F_{1,48} = 20.501$, $P < 0.001$; Figure 1). In contrast, the chemicals induced by jasmonate and herbivory did not respond to BTH. PINII (undetectable expression at all levels) and PPO were not induced by BTH at any concentration (PPO: $R^2 = 0.034$, $F_{1,48} = 1.704$, $P = 0.198$; Figure 1). These chemical responses were mirrored by the bioassays. Applications of BTH caused concentration-dependent reductions in *Pst* lesions ($R^2 = 0.107$, $F_{1,35} = 4.215$, $P = 0.048$; Figure 2). There was no effect of BTH application on the performance of *Trichoplusia ni* caterpillars (RGR: $R^2 = 0.012$, $F_{1,43} = 0.511$, $P = 0.479$, ECI: $R^2 = 0.299$, $F_{1,43} = 1.460$, $P = 0.234$; leaf area consumed: $R^2 = 0.020$, $F_{1,43} = 0.689$, $P = 0.352$; Figure 2).

The protein concentration of leaf tissue was reduced by BTH treatments compared to controls in a concentration dependent manner with the plants at the highest concentration of BTH containing 75% the protein of the control plants ($R^2 = 0.194$, $F_{1,46} = 11.105$, $P = 0.002$; Figure 1).

Time Course. P4 mRNA was present at low levels on the day of 1.2 mM BTH application (0.61 counts), at higher levels on the day after (1.21 counts), and at much higher levels on the fourth (48.52 counts), and eighth days (73.22 counts) ($R^2 = 0.953$, $F_{1,2} = 40.334$, $P = 0.024$).

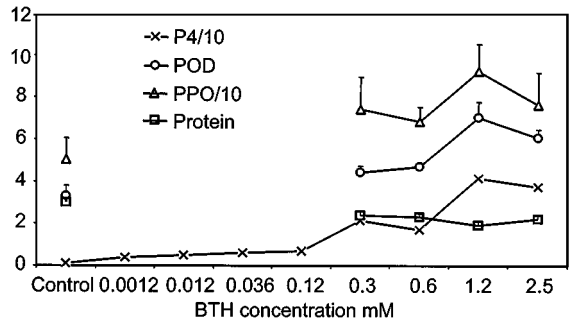


FIG. 1. Effect of different concentrations of BTH (benzothiadiazole) on the activity of chemical constituents of the plant. Relative expression (0-1) of pathogenesis-related protein mRNA expression (P4), peroxidase (POD, ΔOD/g/min), polyphenol oxidase (PPO, ΔOD/g/min), and total protein (mg protein/g tissue). P4 mRNA and POD activity were divided by 10 so they would fit on the same axis.

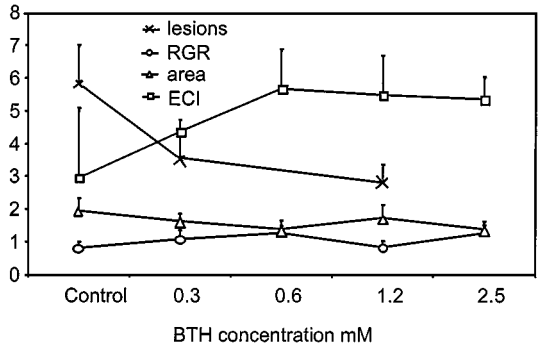
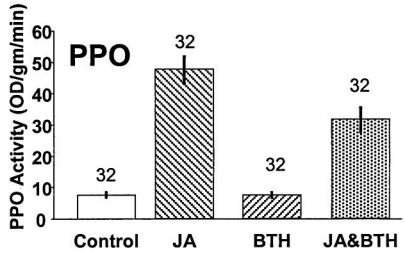


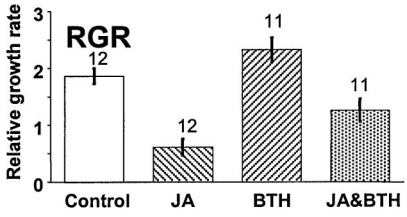
FIG. 2. Effect of different concentrations of BTH (benzothiadiazole) on the performance of the herbivore *Trichoplusia ni* and the pathogen *Pseudomonas syringae* pv. *tomato*. Herbivore performance was measured as relative growth rate (RGR), leaf area consumed (area), and gross growth efficiency (ECI). Performance of the pathogen was measured as the number of lesions formed (lesions). Lesion number was divided by 5 so it would fit on the same axis.

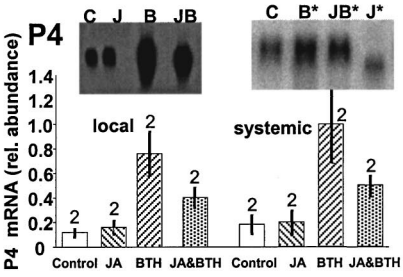
Elicitor Runoff Experiment. Spraying the plants with water at the same time as the JA was sprayed did not alter the effect of JA on PPO activity (mean $\pm$ SE ΔOD/g/min, JA: 17.06 ± 3.80, JA + water: 17.44 ± 5.22; $t = 0.059$, df $= 14$, $P = 0.954$). Spraying the plants with water at the same time as the BTH was sprayed did not change the effect of BTH on P4 mRNA accumulation (mean $\pm$ SE counts: BTH: $185{,}093 \pm 59{,}236$, BTH + water: $128{,}946 \pm 32{,}440$; $t = 0.831$, df $= 4$, $P = 0.453$).

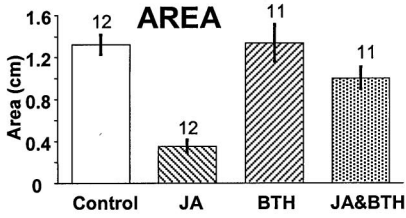
Pathway Interaction Experiments, Biochemical Assays. Attenuation of herbivore responsive proteins in the dual-elicited plants was most consistent when JA and BTH were applied at the same time, regardless of concentration. Application of JA alone increased PPO activity in all experiments (Figures 3–8, Table 1). Application of BTH alone did not affect PPO activity in any of the experiments. The activity of PPO in the dual-elicited plants was reduced when the

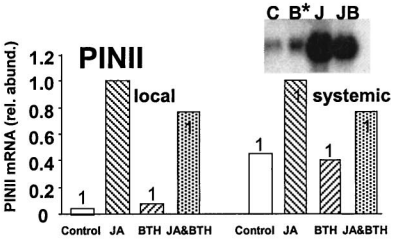
FIG. 3. Chemical and biological measures of resistance for plants in the simultaneous, high-concentration treatment. The four treatments are control plants, plants treated with 1 mM JA, plants treated with 1.2 mM BTH, and plants treated with 1 mM JA and 1.2 mM BTH simultaneously. Chemical measures are polyphenol oxidase (PPO), pathogenesis-related protein 4 (P4), proteinase inhibitor (PINII), peroxidase (POD), and lipoxygenase (LOX). Biological measures are relative growth rate (RGR), leaf area consumed (AREA), and gross growth efficiency (ECI) of the insect *Spodoptera exigua*, and lesion formation (*Pst*) caused by the bacteria *Pseudomonas syringae* pv. *tomato*. Numbers indicate sample size. Inset: representative mRNA blots. *Treatments that are in a different sequence than the labels on the figure axis. Bars indicate mean $\pm$ SE.

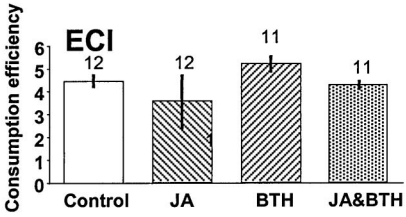

PPO
PPO Activity (OD/gm/min)
Control
JA
BTH
JA&BTH

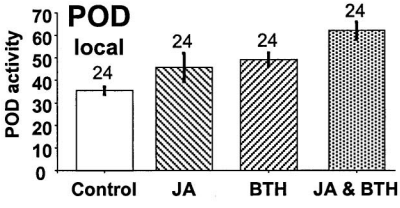

RGR
Relative growth rate
Control
JA
BTH
JA&BTH

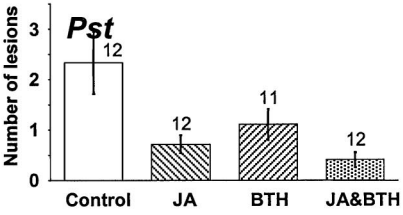

P4
P4 mRNA (rel. abundance)
C J B JB
C B* JB* J*
local
systemic
Control JA BTH JA&BTH Control JA BTH JA&BTH

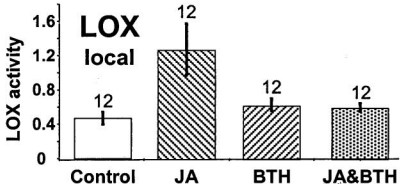

AREA
Area (cm)
Control
JA
BTH
JA&BTH


PINII
PINII mRNA (rel. abund.)
C B* J JB
local
systemic
Control JA BTH JA&BTH Control JA BTH JA&BTH


ECI
Consumption efficiency
Control
JA
BTH
JA&BTH


POD
local
POD activity
Control
JA
BTH
JA & BTH


Pst
Number of lesions
Control
JA
BTH
JA&BTH


LOX
local
LOX activity
Control
JA
BTH
JA&BTH

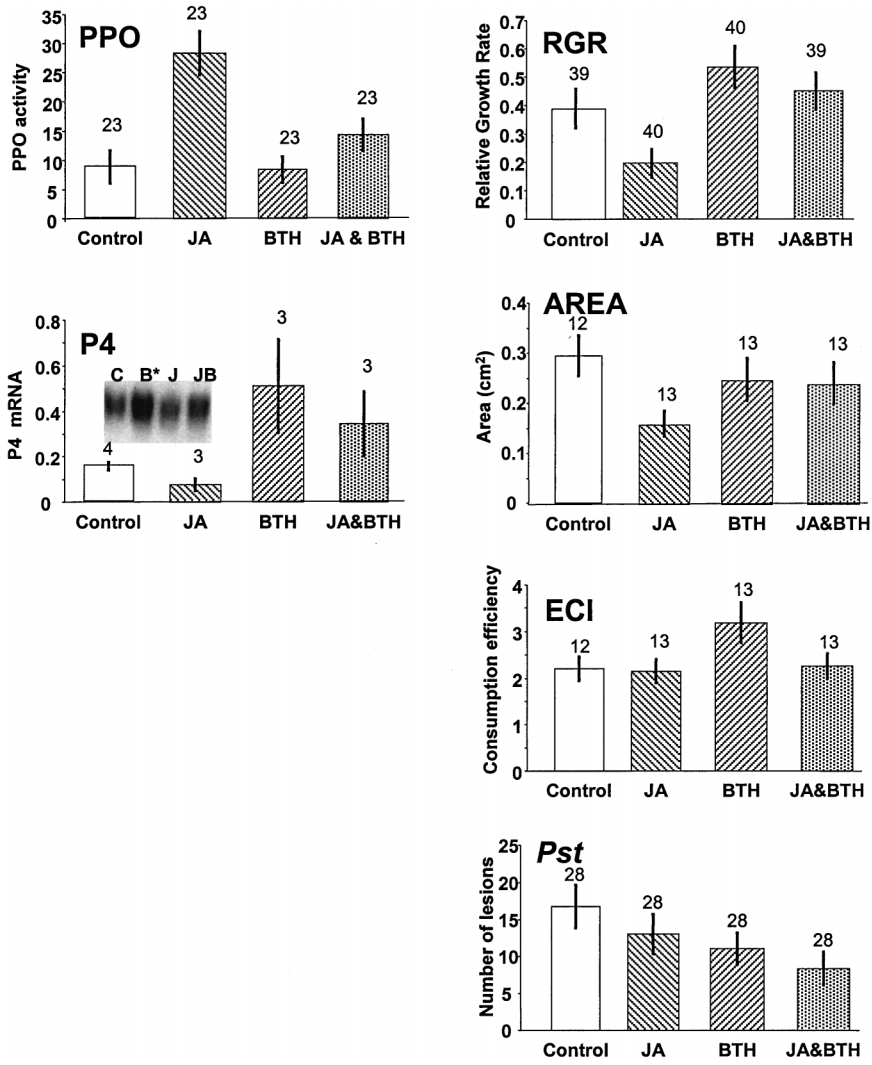


FIG. 4. Chemical and biological measures of resistance for plants in the simultaneous, low-concentration treatment. The four treatments are control plants, plants treated with 0.5 mM JA, plants treated with 0.5 mM BTH, and plants treated with 0.5 mM JA and 0.5 mM BTH simultaneously. Chemical measures are polyphenol oxidase (PPO), and pathogenesis-related protein 4 (P4). Biological measures are relative growth rate (RGR), leaf area consumed (AREA), and gross growth efficiency (ECI) of the insect *Spodoptera exigua*, and lesion formation (*Pst*) caused by the bacteria *Pseudomonas syringae* pv. *tomato*. Numbers indicate sample size. Inset: representative mRNA blot. *Treatments that are in a different sequence than the labels on the figure axis. Bars indicate mean ± SE.

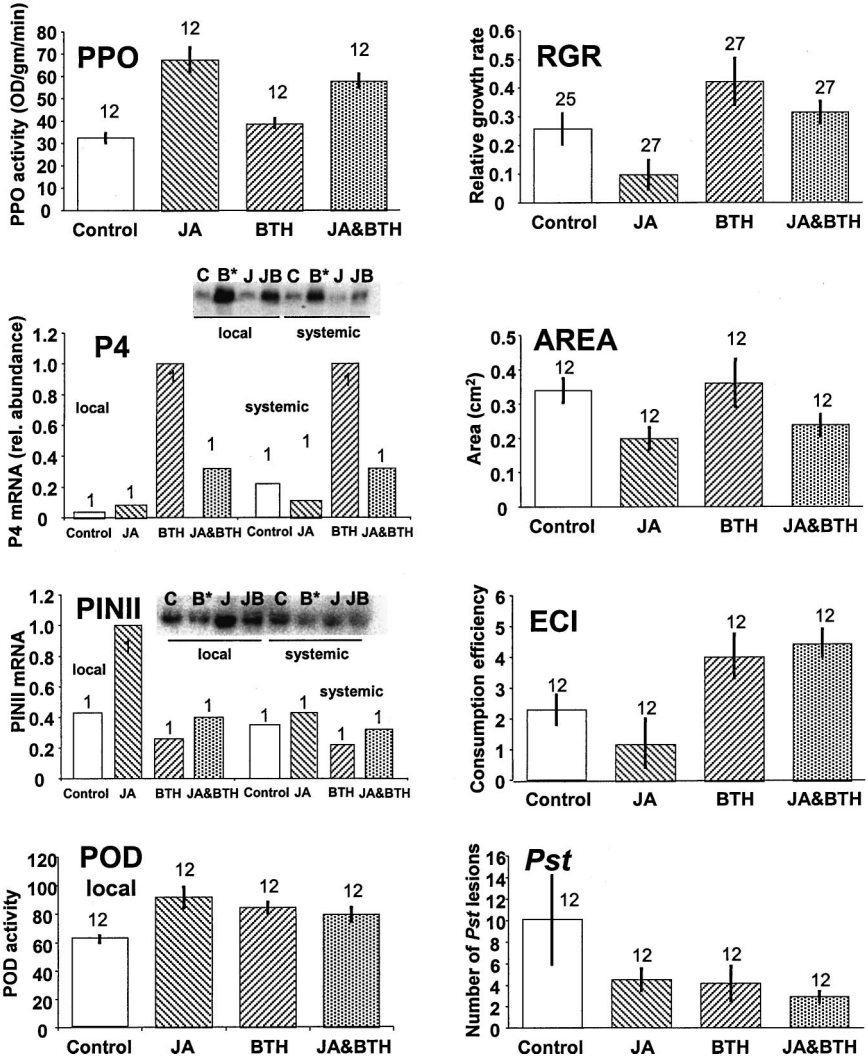


FIG. 5. Chemical and biological measures of resistance for plants in the JA-first, high-concentration treatment. The four treatments are control plants, plants treated with 1 mM JA, plants treated with 1.2 mM BTH, and plants treated with 1 mM JA and then 1.2 mM BTH 2 days later. Chemical measures are polyphenol oxidase (PPO), pathogenesis-related protein 4 (P4), proteinase inhibitor (PINII), and peroxidase (POD). Biological measures are relative growth rate (RGR), leaf area consumed (AREA), and gross growth efficiency (ECI) of the insect *Spodoptera exigua*, and lesion formation (*Pst*) caused by the bacteria *Pseudomonas syringae* pv. *tomato*. Numbers indicate sample size. Inset: representative mRNA blot. *Treatments that are in a different sequence than the labels on the figure axis. Bars indicate mean ± SE.

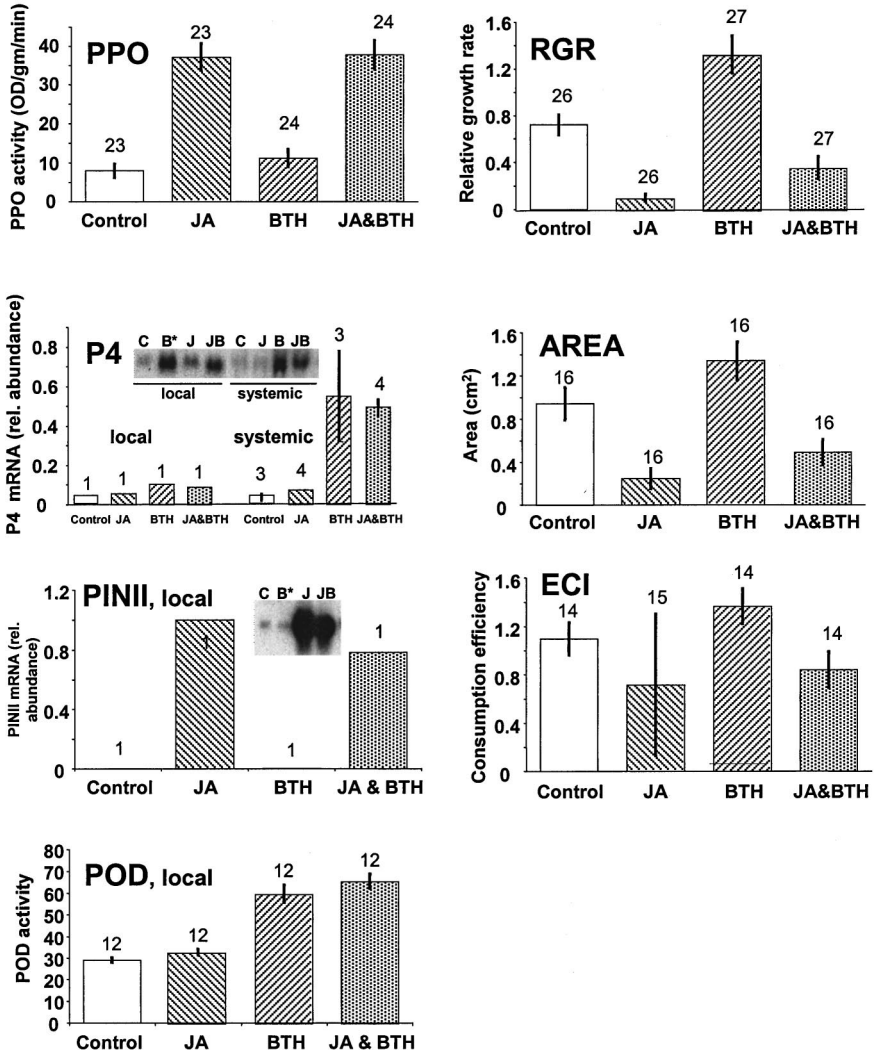


FIG. 6. Chemical and biological measures of resistance for plants in the BTH-first, high-concentration treatment. The four treatments are control plants, plants treated with 1 mM JA, plants treated with 1.2 mM BTH, and plants treated with 1.2 mM BTH and then 1.0 mM JA two days later. Chemical measures are polyphenol oxidase (PPO), pathogenesis-related protein 4 (P4), proteinase inhibitor (PINII), and peroxidase (POD). Biological measures are relative growth rate (RGR), leaf area consumed (AREA), and gross growth efficiency (ECI) of the insect *Spodoptera exigua*, and lesion formation (*Pst*) caused by the bacteria *Pseudomonas syringae* pv *tomato*. Numbers indicate sample size. Inset: representative mRNA blot. *Treatments that are in a different sequence than the labels on the figure axis. Bars indicate mean ± SE.

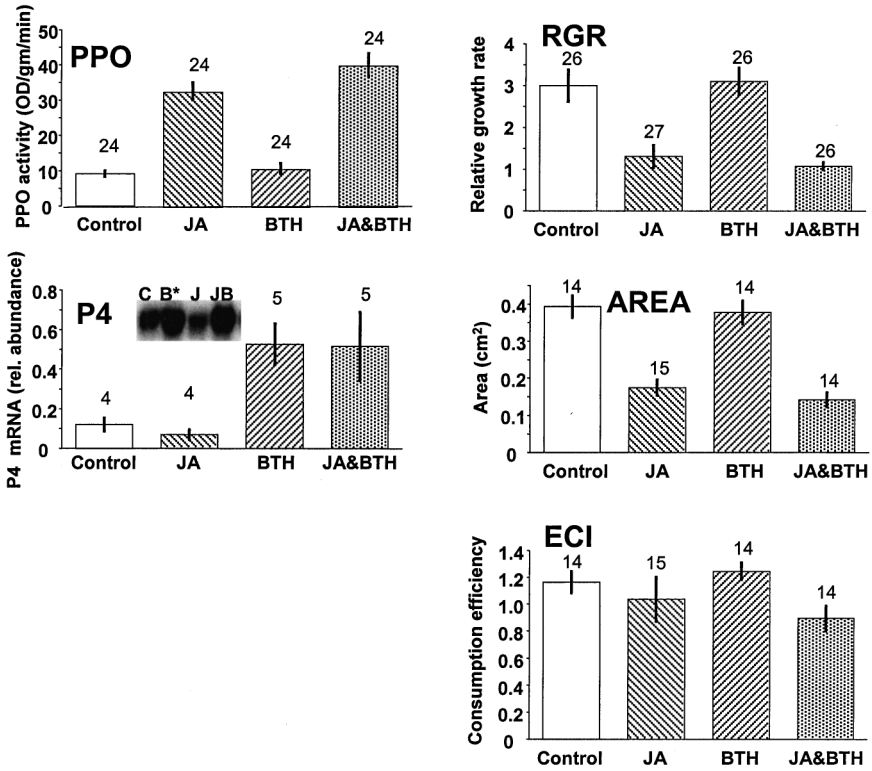


FIG. 7. Chemical and biological measures of resistance for plants in the JA-first, low-concentration treatment. The four treatments are control plants, plants treated with 0.5 mM JA, plants treated with 0.5 mM BTH, and plants treated with 0.5 mM JA and then 0.5 mM BTH 2 days later. Chemical measures are polyphenol oxidase (PPO), and pathogenesis-related protein 4 (P4). Biological measures are relative growth rate (RGR), leaf area consumed (AREA), and gross growth efficiency (ECI) of the insect *Spodoptera exigua*. Numbers indicate sample size. Inset: representative mRNA blot. *Treatments that are in a different sequence than the labels on the figure axis. Bars indicate mean ± SE.

two elicitors were sprayed at the same time compared to plants sprayed with JA alone, at both high and low concentrations (Figures 3 and 4). Activity of PPO was also reduced in the dual-elicited plants when JA was applied first at high concentration (Figure 5), but not when BTH was applied first at the high concentration (Figure 6), or when BTH or JA was applied first at the low concentration (Figures 7 and 8). JA alone increased expression of PINII, and BTH had

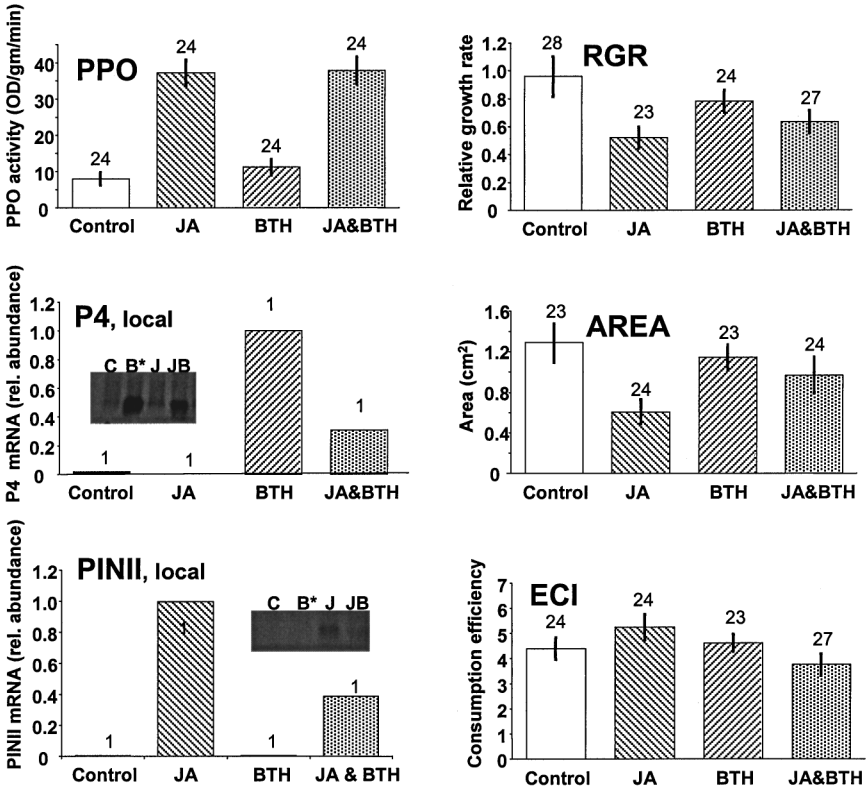


FIG. 8. Chemical and biological measures of resistance for plants in the BTH-first, low-concentration treatment. The four treatments are control plants, plants treated with 0.5 mM JA, plants treated with 0.5 mM BTH, and plants treated with 0.5 mM BTH and then 0.5 mM JA 2 days later. Chemical measures are polyphenol oxidase (PPO), pathogenesis-related protein 4 (P4), and proteinase inhibitor (PINII). Biological measures are relative growth rate (RGR), leaf area consumed (AREA), and gross growth efficiency (ECI) of the insect *Spodoptera exigua*. Numbers indicate sample size. Inset: representative mRNA blot. *Treatments that are in a different sequence than the labels on the figure axis. Bars indicate mean ± SE.

no effect; these results were consistent across experimental trials. Expression of PINII was reduced in all dual-elicited plants, regardless of concentration or timing (Figures 3, 5, 6, and 8). JA alone increased LOX activity, and BTH alone had no effect on LOX in the simultaneous high experiment. Activity of LOX was reduced in dual elicited plants (JA: $F_{1,44} = 5.839$, $P = 0.020$; BTH: $F_{1,44} = 2.691$, $P = 0.1084$; JA × BTH: $F_{1,44} = 6.753$, $P = 0.013$).

TABLE 1. ANOVA ON POLYPHENOL OXIDASE ACTIVITY (ΔOD/g/min) OF PLANTS IN CONTROL, JA, BTH AND JA BTH AND GROUPS IN SIX CONCENTRATION (LOW = 0.5 mM JA, 0.3 mM BTH AND HIGH = 1.5 mM JA AND 1.2 mM BTH) AND TIMING (SIMULTANEOUS AND 2-DAY DELAYED ELICITATION) COMBINATIONS[a]

Experiment	Source	*df*	*F*	*P*
Simultaneous high	JA	1	276.765	<0.001
	BTH	1	6.688	0.011
	Trial	2	30.746	<0.001
	JA × BTH	1	7.802	0.006
	JA × trial	2	37.915	<0.001
	Error	116		
Simultaneous low	JA	1	18.684	<0.001
	BTH	1	6.025	0.018
	JA × BTH	1	5.315	0.026
	Error	88		
JA first high	JA	1	77.158	<0.001
	BTH	1	0.080	0.779
	JA × BTH	1	6.259	0.016
	Error	44		
BTH first high	JA	1	126.351	<0.001
	BTH	1	0.651	0.422
	Trial	1	33.836	<0.001
	JA × BTH	1	0.150	0.700
	Error	86		
JA first low	JA	1	148.910	<0.001
	BTH	1	1.475	0.228
	Trial	1	7.063	0.009
	JA × BTH	1	0.493	0.484
	Error	88		
BTH first low	BTH	1	0.146	0.703
	JA	1	0.146	<0.001
	Trial	1	2.761	0.100
	JA × BTH	1	0.375	0.542
	Error	116		

[a] Trial is included as a factor in the experiments that were conducted more than once. All interactions were included in the model; non-significant interactions are not reported.

Attenuation in expression of BTH-responsive genes was less pronounced than the attenuation of JA-responsive proteins. Across all experiments, BTH alone increased expression of P4 mRNA accumulation, and JA had no effect (Figures 3–8, Table 2). Accumulation of P4 mRNA was not reduced in dual-elicited plants compared to BTH alone, although there was a non statistically significant trend in this direction in most of the experiments. This trend was observed in the simultaneous elicitation at both high and low concentration experiments: the high concentration

TABLE 2. ANOVA ON PATHOGENESIS-RELATED PROTEIN 4 mRNA EXPRESSION OF PLANTS IN CONTROL, JA, BTH, AND JA AND BTH GROUPS IN FOUR OF SIX CONCENTRATION (LOW = 0.5 mM JA, 0.3 mM BTH AND HIGH = 1.5 mM JA AND 1.2 mM BTH) AND TIMING (SIMULTANEOUS AND 2-DAY DELAYED ELICITATION) COMBINATIONS[a]

Experiment	Source	*df*	*F*	*P*
Simultaneous high	JA	1	0.360	0.560
	BTH	1	24.120	<0.001
	JA × BTH	1	1.773	0.208
	Error	12		
Simultaneous low	JA	1	0.902	0.363
	BTH	1	5.850	0.034
	JA × BTH	1	0.031	0.863
	Error	11		
BTH first high	JA	1	0.008	0.930
	BTH	1	7.464	0.021
	JA × BTH	1	0.084	0.777
	Error	10		
JA first low	JA	1	0.229	0.639
	BTH	1	23.891	<0.001
	JA × BTH	1	0.049	0.827
	Error	14		

[a] Local and systemic samples were grouped for statistics. The two combinations not reported below were not statistically analyzed because only one replicate of each treatment was obtained.

of JA applied first, and experiments with the low concentration of BTH applied first. The P4 activity between the BTH alone and the BTH and JA treated plants was nearly identical in the high concentration BTH first and the low concentration JA first experiments. Activity of POD increased in local leaves sprayed with JA and BTH singly and these effects were not altered by dual spray at high concentration regardless of timing (Figures 3–5, Table 3); POD was not examined at low-concentration sprays.

Pathway Interaction Experiments: Bioassays. Like the chemical measures of resistance, attenuation of plant resistance to herbivores was consistently seen when JA and BTH were applied at the same time, regardless of concentration. Attenuation of resistance to herbivores was also seen in the high-concentration delayed-spray treatments, but not in the low-concentration delayed-spray treatments. Relative growth rate (RGR) and amount of foliage consumed (AREA) were influenced by elicitor treatment, whereas gross growth efficiency (ECI) was not. Applications of JA by itself reduced the RGR of *S. exigua* in all experiments (Figures 3–8, Table 4). BTH by itself increased the RGR of *S. exigua* consistently in high-concentration experiments, but only in the simultaneous elicitation low-concentration experiment. At the high concentrations of JA and BTH, RGR of *S. exigua* increased on dual-sprayed plants compared to plants sprayed with JA alone. At the low

TABLE 3. ANOVA ON LOCAL PEROXIDASE ACTIVITY (ΔOD/g/min) OF PLANTS IN CONTROL, JA, BTH AND JA BTH AND GROUPS IN THREE HIGH CONCENTRATION (1.5 mM JA AND 1.2 mM BTH) AND TIMING (SIMULTANEOUS AND 2-DAY DELAYED ELICITATION) COMBINATIONS[a]

Experiment	Factor	*df*	*F*	*P*
Simultaneous high	JA	1	3.643	0.060
	BTH	1	32.361	0.000
	Trial	1	1.309	0.256
	JA × BTH	1	1.972	0.164
	JA × trial	1	5.944	0.017
	Error	88		
JA first high	JA	1	5.590	0.023
	BTH	1	0.822	0.370
	JA × BTH	1	11.246	0.002
	Error	44		
BTH first high	JA	1	3.026	0.089
	BTH	1	133.260	0.000
	JA × BTH	1	0.171	0.681
	Error	44		

[a] All interactions were included in the model; non-significant interactions are not reported.

concentration, the increase in RGR in the dual-sprayed plants was only seen when the elicitors were sprayed simultaneously.

JA alone reduced AREA in all experiments (Figures 3–8, Table 5). BTH alone increased AREA in the BTH-first, high-concentration experiment, but in the other experiments had no effect when applied singly. Dual-elicitation increased the amount of foliage consumed compared to the JA-treated plants in the simultaneous-elicitation experiments at both concentrations and when BTH was sprayed before JA at both concentrations. For dual elicitation to affect AREA, either a high concentration of BTH must be applied or BTH must be applied before the JA. JA tended to decrease ECI and BTH tended to increase ECI, but these patterns were only significant in a few cases (Figures 3–8, Table 6). JA and BTH did not interact in their effects on ECI. Effects on RGR and AREA but not ECI indicate that herbivore rate of consumption (and thus growth) were affected by JA, but the ability of the herbivore to convert food into herbivore biomass was not affected.

Pathway Interaction Experiments: Disease Assay. BTH consistently reduced the number of *Pst* lesions while JA was inconsistent in this effect (Figures 3–5, Table 7). Elicitation by JA alone reduced the number of lesions in the simultaneous high-concentration experiments, but not in the simultaneous low- or JA-first high-concentration experiment. When both elicitors were applied in the simultaneous high- and simultaneous low-concentration experiments, the fewest lesions were

TABLE 4. ANOVA ON RELATIVE GROWTH RATE [(FINAL WEIGHT-INITIAL WEIGHT)/(INITIAL WEIGHT)] OF *Spodoptera exigua* CATERPILLARS FEEDING ON PLANTS IN CONTROL, JA, BTH, AND JA BTH AND GROUPS IN SIX CONCENTRATION (LOW = 0.5 mM JA, 0.3 mM BTH AND HIGH = 1.5 mM JA AND 1.2 mM BTH) AND TIMING (SIMULTANEOUS AND 2-DAY DELAYED ELICITATION) COMBINATIONS[a]

Experiment	Source	*df*	*F*	*P*
Simultaneous high	JA	1	43.457	<0.001
	BTH	1	9.956	0.003
	JA × BTH	1	0.290	0.593
	Error	42		
Simultaneous low	JA	1	5.308	0.023
	BTH	1	12.032	0.001
	JA × BTH	1	0.311	0.578
	Trial	3	16.487	<0.001
	BTH × trial	3	4.754	0.003
	Error	141		
JA first high	JA	1	5.485	0.024
	BTH	1	11.096	0.002
	JA × BTH	1	0.230	0.634
	Error	44		
BTH first high	JA	1	89.079	<0.001
	BTH	1	18.443	<0.001
	Trial	1	11.230	0.001
	JA × BTH	1	1.287	0.259
	Error	98		
JA first low	JA	1	97.926	<0.001
	BTH	1	0.165	0.685
	Trial	1	100.278	<0.001
	JA × BTH	1	0.965	0.328
	JA × trial	1	24.886	<0.001
	BTH × trial	1	5.243	0.024
	Error	98		
BTH first low	JA	1	10.127	0.002
	BTH	1	0.008	0.929
	Trial	1	13.002	0.001
	JA × BTH	1	2.883	0.093
	JA × BTH × trial	1	8.076	0.006
	Error	97		

[a] Trial is included as a factor in the experiments that were conducted more than once. All interactions were included in the model; non-significant interactions are not reported.

found on the dual-elicited plants compared to singly elicited plants or controls. In the JA first high-concentration experiment, the dual-elicited plants had the same number of lesions as the plants only elicited with BTH.

S. exigua Performance on Wild-Type and Jasmonate-Deficient Plants. *S. exigua* mass was increased on wild-type plants treated with BTH but not on

TABLE 5. ANOVA ON LEAF AREA EATEN (mm^2) BY *Spodoptera exigua* CATERPILLARS FEEDING ON PLANTS IN CONTROL, JA, BTH, AND JA BTH AND GROUPS IN SIX CONCENTRATION (LOW = 0.5 mM JA, 0.3 mM BTH AND HIGH = 1.5 mM JA AND 1.2 mM BTH) AND TIMING (SIMULTANEOUS AND 2-DAY DELAYED ELICITATION) COMBINATIONS[a]

Experiment	Source	*df*	*F*	*P*
Simultaneous high	JA	1	30.001	0.000
	BTH	1	7.744	0.008
	JA × BTH	1	7.007	0.001
	Error	41		
Simultaneous low	JA	1	3.632	0.063
	BTH	1	0.183	0.670
	JA × BTH	1	2.830	0.099
	Error	47		
JA first high	JA	1	9.153	0.004
	BTH	1	0.344	0.561
	JA × BTH	1	0.140	0.710
	Error	44		
BTH first high	JA	1	39.955	<0.001
	BTH	1	5.546	0.022
	JA × BTH	1	0.004	0.948
	Error	60		
JA first low	JA	1	80.061	0.000
	BTH	1	0.989	0.325
	JA × BTH	1	0.176	0.675
	Error	53		
BTH first low	JA	1	12.428	0.001
	BTH	1	2.353	0.129
	Trial	1	38.848	<0.001
	JA × BTH	1	6.111	0.015
	JA × BTH × trial	1	4.902	0.029
	Error	86		

[a] Trial is included as a factor in the experiments that were conducted more than once. All interactions were included in the model; non-significant interactions are not reported.

the jasmonate-deficient plants treated with BTH (wild-type: BTH $F_{1,57} = 10.53$, $P = 0.002$, trial $F_{2,57} = 29.22$, $P < 0.001$; jasmonate-deficient: BTH $F_{1,58} = 0.028$, $P = 0.86$, trial $F_{2,59} = 72.19$, $P < 0.001$; Figure 9). More caterpillars died on the wild-type plants compared to the wild-type + BTH-treated plants (8/33 wild type, 2/35 wild type + BTH; $G = 3.7$, $P = 0.054$). There was no detectable difference in caterpillar mortality on the jasmonate-deficient plants (1/32 def-1, 4/33 def-1 + BTH; $G = 1.71$, $P = 0.19$). A functional jasmonate pathway is required for the positive effect of BTH on caterpillar performance, indicating that the mechanism of BTH action is through interference with the jasmonate pathway.

TABLE 6. ANOVA ON GROSS GROWTH EFFICIENCY OF *Spodoptera exigua* CATERPILLARS FEEDING ON PLANTS IN CONTROL, JA, BTH, AND JA BTH AND GROUPS IN SIX CONCENTRATION (LOW = 0.5 mM JA, 0.3 mM BTH AND High = 1.5 mM JA AND 1.2 mM BTH) AND TIMING (SIMULTANEOUS AND 2-DAY DELAYED ELICITATION) COMBINATIONS[a]

Experiment	Source	*df*	*F*	*P*
Simultaneous high	JA	1	2.432	0.126
	BTH	1	1.520	0.224
	JA × BTH	1	0.000	0.985
	Error	42		
Simultaneous low	JA	1	2.806	0.101
	BTH	1	2.393	0.129
	JA × BTH	1	1.463	0.232
	Error	47		
JA first high	JA	1	0.102	0.750
	BTH	1	6.018	0.018
	JA × BTH	1	0.550	0.462
	Error	44		
BTH first high	JA	1	6.628	0.013
	BTH	1	0.631	0.430
	JA × BTH	1	0.194	0.661
	Error	57		
JA first low	JA	1	5.780	0.020
	BTH	1	0.106	0.746
	JA × BTH	1	1.260	0.267
	Error	53		
BTH first low	JA	1	0.017	0.896
	BTH	1	2.359	0.128
	Trial	1	2.451	0.121
	JA × BTH	1	4.320	0.041
	Error	90		

[a] Trial is included as a factor in the experiments that were conducted more than once. All interactions were included in the model; non-significant interactions are not reported.

DISCUSSION

Our study has five key findings: (1) antagonism between the JA and SA pathways is observed in the both chemical and biological assays, although this was asymmetric, with the effects of BTH on the jasmonate pathway being more frequent than the effects of JA on the salicylate pathway. (2) Timing of elicitation influenced chemical responses and biological resistance, with simultaneous elicitation consistently showing a negative signal interaction. (3) Concentration of elicitation was most important in determining the chemical attenuation, with high concentration of elicitors consistently resulting in a negative interaction. (4) Temporal separation and/or low concentration of elicitors may reduce the negative signal

TABLE 7. ANOVA FOR *Pst* LESIONS[a]

Experiment	Source	*df*	*F*	*P*
Simultaneous high	JA	1	11.032	0.001
	BTH	1	4.794	0.031
	Trial	1	5.330	0.023
	JA × BTH	1	1.757	0.188
	Error	87		
Simultaneous low	JA	1	2.535	0.114
	BTH	1	7.953	0.006
	Trial	2	13.827	<0.001
	JA × BTH	1	0.133	0.716
	BTH × trial	2	5.864	0.004
	Error	100		
JA first high	JA	1	0.934	0.339
	BTH	1	4.176	0.047
	JA × BTH	1	0.980	0.328
	Error	44		

[a] ANOVA on the number of lesions formed by *Pseudomonas syringae* pv. *tomato* growing on plants in the Control, JA, BTH and JA BTH and groups in the high and low simultaneous elicitation experiments (low = 0.5 mM JA, 0.3 mM BTH and high = 1.5 mM JA and 1.2 mM BTH) and in the experiment where high concentration JA was applied 2 days before high concentration BTH. Trial is included as a factor in the experiments that were conducted more than once. All interactions were included in the model; non-significant interactions are not reported.

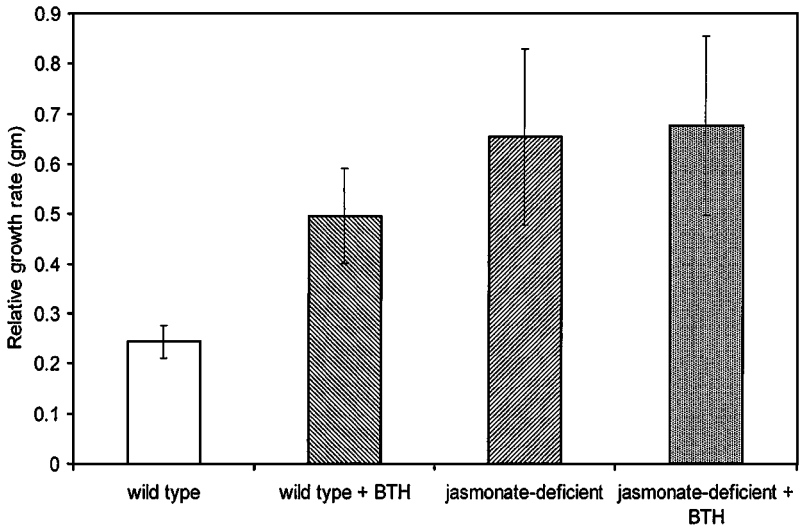


FIG. 9. Relative growth rate of *Spodoptera exigua* caterpillars [(final weight − initial weight)/(initial weight)] feeding on plants in four treatments: wild-type, wild-type treated with 1.2 mM benzothiodiazole (BTH), jasmonate-deficient, and jasmonate-deficient treated with 1.2 mM BTH. Bars indicate mean ± SE.

TABLE 8. SUMMARY OF ATTENUATION OF CHEMICAL EXPRESSION OF JASMONIC ACID AND SALICYLIC ACID PATHWAYS IN DUAL-ELICITED PLANTS[a]

	Simultaneous					JA first				BTH first			
	PPO	PINII	LOX	P4	POD	PPO	PINII	P4	POD	PPO	PINII	P4	POD
Low	Y	NT	Y	Y	NT	N	NT	N	NT	N	Y	Y	NT
High	Y	Y	Y	Y	N	Y	Y	Y	N	N	Y	N	N

[a] Markers of the jasmonate pathway are polyphenol oxidase (PPO), proteinase inhibitors (PINII), and lipoxygenase (LOX). Markers of the salicylate pathway are pathogenesis-related protein 4 (P4), and peroxidase (POD). Y = significant attenuation in the dual elicited plants compared to the singly elicited plants, N = no attenuation, NT = not tested.

interaction. (5) The negative signal interaction was observed more consistently in biological assays than in chemical assays, underscoring the importance of linking the chemistry of the putative plant defense with their apparent targets.

Effects of BTH on Plant Responses and Herbivore Performance. Elicitation of tomato plants by BTH affected production of primary and secondary plant metabolites. BTH induced the pathogenesis-related protein, P4, in a concentration-dependent manner starting two days after application and lasting for at least eight days. BTH treatment at the highest concentration reduced total protein concentration approximately 20% compared to the control. This is in contrast to the results of Inbar et al. (1998), who found no effect of BTH application on foliar protein in tomato. Thus, we found no support for the hypothesis that BTH elicitation benefits herbivores by increasing plant protein.

Effects of Concentration and Timing of Elicitors on Antagonistic Interactions Between Plant Defense Pathways. Concentration and timing of elicitation affected JA-mediated chemical responses and biological resistance in overlapping but not identical ways (Tables 8 and 9). The negative signaling interaction between the jasmonate and salicylate responses was seen in PPO and PINII, hallmark responses

TABLE 9. SUMMARY OF ATTENUATION OF BIOLOGICAL RESISTANCE TO HERBIVORES AND PATHOGENS IN DUAL-ELICITED PLANTS[a]

	Simultaneous				JA first				BTH first			
	RGR	AREA	ECI	Lesions	RGR	AREA	ECI	Lesions	RGR	AREA	ECI	Lesions
Low	Y	Y	N	N	N	N	N	NT	N	Y	N	NT
High	Y	Y	N	N	Y	N	Y	N	Y	Y	N	NT

[a] The measures of performance of the herbivore, *Spodoptera exigua*, were relative growth rate [RGR, (final weight - initial weight)/(initial weight)], leaf area eaten (AREA, mm^2 of foliage consumed), gross growth efficiency [ECI, (final weight - initial weight)/(area consumed)]. Y = significant attenuation in the dual elicited plants compared to the singly elicited plants, N = no attenuation, NT = not tested.

of the jasmonate pathway in tomato as well as herbivore performance. For PPO, the timing of the elicitors was important; the attenuation of induction was most consistently seen when the compounds were applied at the same time, irrespective of concentration. The attenuation in PINII expression was consistent, regardless of concentration–timing combination. Concentration of elicitors was only important for relative growth rate of *S. exigua* when the elicitor applications were separated in time. At high concentration, there was always attenuation, regardless of timing. At low concentration elicitation, there was only attenuation when the plants were elicited simultaneously, not when the applications were separated in time.

Pst was negatively affected by both JA and BTH, with no attenuation of resistance in the dual-elicited treatments. *Pst* is a complicated pathogen because it triggers SAR and jasmonate defenses through its release of coronatine into the leaf. Given that the JA and SA pathways interact with each other, even prior to exogenous induction, it is difficult to estimate the relative roles of the pathways on *Pst* performance.

Does Temporally Separating Elicitation of Pathways or Decreasing Concentration of Elicitor Reduce Negative Signal Interaction? In the broad sense, yes, the antagonism was less predictable in the experiments where the elicitors were used at low concentration and/or were separated in time. However, the predictions do not hold for each particular concentration and timing combination, and chemical attenuation does not always match the biological attenuation of resistance to herbivores. Our original hypotheses were based on the inhibition between the two pathways due to the action of JA and SA molecules per se. A complete understanding of how these interact awaits further discoveries of the components in each signaling pathway (Felton and Korth, 2000; McDowell and Dangl, 2000). Other plant hormones, such as ethylene, could also influence the interaction between the jasmonate and salicylate pathways (O'Donnell et al., 1996; Zhu-Salzman et al., 1998).

There was a greater effect of BTH on jasmonate-mediated responses than of JA on BTH-mediated responses. However, the trends for attenuation of P4 expression were similar to the trends for PPO activity. During simultaneous elicitation, attenuation is obtained regardless of elicitor concentration, but during temporally separated elicitation, elicitor concentration does influence whether P4 expression is attenuated in the dual-elicited plants. In contrast to these non statistically significant effects, Thaler et al. (1999) found attenuation of BTH-induced P4 by jasmonate spray. The Thaler et al. (1999) study differed from this work in several ways: the experiments were conducted in field-grown plants, entire plants were treated with both elicitors, and larger sample sizes were employed, allowing detection of smaller effects. The data presented here suggest that JA attenuates BTH action. However, this could also be partially explained by elicitor runoff. While the reduction in P4 expression in the BTH + water compared to the BTH-alone treatment was not significant, the reduction was of the same magnitude as when P4 expression in the BTH + JA and BTH-alone treatments are compared.

Paths of Signal Interaction. The signal interaction between the JA and SA pathways was detected acting through two distinct paths. In the first path (nonadditive) of antagonism between the JA and SA pathway, there was a statistically significant interaction between the effects of JA and BTH. For example, JA singly induced PPO, BTH singly had no effect on PPO, and there was reduced PPO activity in dual-elicited plants compared to plants elicited with JA alone (e.g., Figure 5, PPO; Table 1). In this case, BTH had no effect on constitutive PPO activity by itself, but interfered with the induction of the jasmonate pathway when JA was applied to the plant. These are nonadditive effects and reflect the more straightforward signal interactions reported in the literature where SAR activation reduces the expression of JA-mediated responses (indicated by PPO activity). Our elicitor runoff experiments suggest that this is an in vivo interaction and not an effect of the elicitors at the plant surface.

The second path (additive) occurred when the main effects of JA and BTH were both significant, with the means in opposite directions, and the interaction term was not significant. For example, JA singly decreased RGR, BTH singly increased RGR, and the RGR of herbivores on dual-elicited plants was intermediate (e.g., Figure 3, RGR; Table 4). In this case, PPO activity was not reduced in the BTH single elicitor treatment, but elicitation by BTH still had a positive effect on RGR. The effects of JA and BTH on RGR were additive but appeared independent of the PPO correlated suite of responses in tomato plants. In all of the timing–concentration scenarios where attenuation was seen in the dual-elicited plants, PPO activity was influenced via pathway 1 (nonadditively) and caterpillar RGR via pathway 2 (additively).

What Aspect of SAR Results in Attenuation of Caterpillar RGR? Previous work has established PPO activity in tomato plants as a good predictor of caterpillar performance (Broadway et al., 1986; Felton et al., 1989; Duffey and Felton, 1991; Stout et al., 1998). In this study, PPO activity is negatively correlated with herbivore performance with some exceptions. When we take the entire data set from this study and examine the relationship between PPO activity and RGR (the mean PPO and RGR from each treatment group in each experiment is the unit of replication), we find that PPO and RGR are inversely correlated ($F_{1,30} = 4.45$, $P = 0.041$, $R^2 = 0.13$). This indicates that increased PPO activity is correlated with reduced the performance of herbivores but that other jasmonate-related compounds not correlated with PPO activity are also important.

Many studies have found that SAR activation increases the performance of herbivores. In this study, we generally find that SAR activation increases the RGR of *S. exigua* caterpillars both when SAR is induced singly and when SAR and JA responses are induced in combination. Elicitation of SAR itself never reduced constitutive PPO activity, while it did increase the RGR of *S. exigua*. In dual SAR/JA-elicited plants in many experiments, SAR elicitation decreased PPO activity but it only increased RGR in the experiments where SAR elicitation alone

increased RGR. This suggests that SAR elicitation increases caterpillar performance independent of PPO correlated jasmonate responses. However, SAR elicitation increased caterpillar growth rate on the wild-type plants, but not on the jasmonate-deficient plants. This indicates that SAR elicitation does increase caterpillar growth through a jasmonate-dependent mechanism, possibly by preventing induction of the jasmonate pathway by the caterpillar during the three-day assay. At this stage we cannot distinguish between PPO simply being a variable correlate of JA-mediated responses and BTH preventing induction of PPO correlated responses that are induced when the bioassay organism is feeding on the plant.

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FLORAL SYNOMONE OF A WILD ORCHID, *Bulbophyllum cheiri*, LURES *Bactrocera* FRUIT FLIES FOR POLLINATION

KENG-HONG TAN,[1,*] RITSUO NISHIDA,[2] and YOCK-CHAI TOONG[3]

[1]*School of Biological Sciences*
Universiti Sains Malaysia
11800, Penang, Malaysia

[2]*Laboratory of Chemical Ecology*
Graduate School of Agriculture, Kyoto University
Kyoto 606-8502, Japan

[3]*School of Chemical Sciences*
Universiti Sains Malaysia
11800, Penang, Malaysia

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Abstract—The major fruit fly attractant component in the floral fragrance of *Bulbophyllum cheiri* (fruit fly orchid) is methyl eugenol (ME). In the lowland rain forest of Malaysia, the solitary and nonresupinate flowers of the fruit fly orchid attract only males of the ME-sensitive fruit fly species (*Bactrocera carambolae*, *B. papayae*, and *B. umbrosa*. During the morning, the fruit fly orchid flower is visited by many fruit flies, which can sometimes cover the whole flower. The number of visitors dwindles in the afternoon. Headspace analysis of the flower shows a high ME peak in the morning, a small one between 12:00 and 14:00 hr, and no detectable ME peak after 14:00 hr. The process of pollination in the wild is initiated by attraction of fruit flies to floral ME. The flower, with the aid of its specialized hinged see-saw lip (labellum), temporarily traps (<1 min) a fruit fly pollinator between its lip and column. Just prior to this, the fly is rewarded by the opportunity to feed on the floral attractant found on surfaces of petals, sepals, and lip. The pollinaria borne by two wild *B. papayae* males (caught on and near the fruit fly orchid flower) are identical in morphology and structure with those obtained from the flower. Many of the *B. papayae* males (17 of 22 analyzed) attracted to the fruit fly orchid already possessed both ME metabolites, *trans*-coniferyl alcohol and 2-allyl-4,5-dimethoxyphenol, in their rectal glands, indicating that they had previously consumed ME. In this orchid–fruit fly association, both organisms gain direct reproductive benefits: the orchid flower gets pollinated without having to offer nectar, while the fruit fly boosts its pheromone and defense system, as well as its sexual competitiveness by feeding on the ME produced by the flower.

* To whom correspondence should be addressed. E-mail: tan_kh@tm.net.my

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Key Words—*Bulbophyllum cheiri*, fruit fly orchid, Orchidaceae, fruit flies, Tephritidae *Bactrocera* spp., methyl eugenol, floral scent, pheromone, synomone, pollination, coevolution.

INTRODUCTION

Many species of wild orchids have myophilous (fly pollinated) flowers. The genus *Bulbophyllum* (Orchidaceae) is characterized by its well-balanced see-saw lip (labellum), which is attached to the base of the floral column by a small springy hinge. The flowers produce foul-smelling scent to attract flies (Van der Pijl and Dodson, 1969). Of the many myophilous orchids, however, the involvement of tephritid fruit flies in pollination has not been mentioned (Kevan and Baker, 1983; Metcalf and Metcalf, 1992). Several species of *Bulbophyllum* are pollinated by flies (Christensen, 1994). In Brazil, milichiid female flies (*Pholeomyia* spp., Diptera: Milichiidae) pollinate three species—*Bu. involutum* Borba, Semir and F. Barros, *Bu. ipanemense* Hoehne, and *Bu. weddellii* (Lindl.) Rchb. f. (Borba and Semir, 1998b). In Queensland, *Bu. baileyi* F. Müell. flowers have a fruity odor (chemical identity unknown) and are reportedly visited by fruit flies—*Strumeta* (currently under *Bactrocera*) species (Symthe, 1969). More recently, it was shown that males of many fruit fly species [*Bactrocera* (formerly *Dacus*) spp., Diptera: Tephritidae] are attracted to flowers of the ginger orchid (*Bulbophyllum patens* King), particularly to the ginger essence of the floral scent zingerone, where they perform pollination. Zingerone attracts both the methyl eugenol (ME) attracted and the raspberry ketone (RK) attracted fruit fly species (Tan and Nishida, 2000). In order to better understand the coevolution between *Bulbophyllum* and fruit flies, it is necessary to search systematically for, and investigate, orchid flowers that release either ME or RK specifically to attract the male *Bactrocera* flies sensitive to these floral scent components.

One orchid species that has yet to be studied is *Bulbophyllum cheiri* Lindley (henceforth referred to as fruit fly orchid) that is found primarily in lowland rain forest of Malaysia. It belongs to the genus *Bulbophyllum* sect. Sestochilus (Breda) Benth. & Hook. f., and comprises 60–70 species worldwide. *Bu. cheiri* bears solitary flowers that resemble a closed hand with fingers almost meeting at the tips (Figure 1A) (Vermeulen, 1991). It possesses a slender lip that has a broad base, which is hinged to the column. In the wild, the flower is postured in order to position the movable see-saw lip in a higher plane than the column—a characteristic of a nonresupinate (not upside down) orchid flower. The flower is visited by fruit flies as soon as it blooms (Figure 1A) and occassionally has been observed to be completely covered with fruit flies. However, it never attracts other insects.

Males of *Bactrocera dorsalis* Hendel, *B. carambolae* Drew & Hancock, *B. papayae* Drew & Hancock, and *B. umbrosa* Fabricius are all attracted to ME and are known to be serious pests to many fruit species in tropical and subtropical

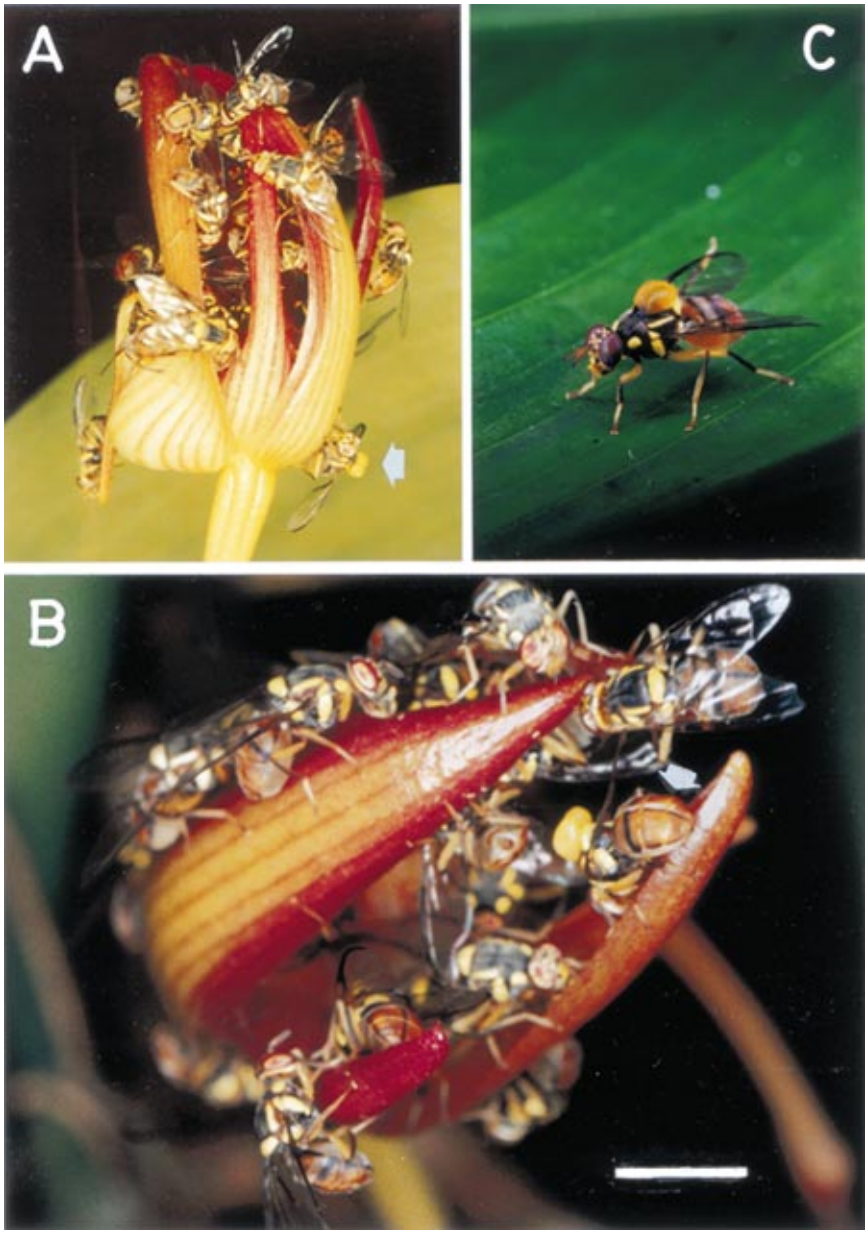


FIG. 1. (A) Male fruit flies of *Bactrocera papayae* lapping on synomone containing methyl eugenol of a fully blossomed flower of the fruit fly orchid (*Bulbophyllum cheiri*). Note: a fruit fly bearing a pollinarium at the base of the medial sepal (white arrow); and a fruit fly facing downwards on the slender tip of the movable see-saw lip (left bottom). (B) A close-up view of fruit flies lapping on the surface of *Bulbophyllum cheiri* flower. Note: posterior view of a male of *Bactrocera papayae* bearing a pollinarium on a lateral sepal (white arrow). Bar = 5.0 mm. (C) A frontal view of a male *Bactrocera papayae* bearing a pollinarium of *Bulbophyllum cheiri* consisting of two pairs of pollinia of approximately equal length, on its thoracic dorsum (The pollinarium remained stuck to the fly for over one month until death in captivity).

regions. When males are fed ME from either natural or synthetic sources, they convert it into its analog(s), which are used to boost their sex pheromone system (Tan and Nishida, 1996). Over 200 species from as many as 32 plant families have been reported to contain ME in shoots, blossoms and/or leaves (Tan and Toong, unpublished data).

Unlike many other orchids, the flower of the fruit fly orchid does not produce nectar to attract bees, wasps, or butterflies, but instead attracts only certain species of *Bactrocera* fruit flies through a mildly scented fragrance resembling that of ME. This paper reports the results of a study on *Bu. cheiri* and its association with fruit flies. The research had three goals: (a) chemical analysis of the attractant in the flower that lures fruit flies, (b) behavioral observations of fruit flies on the orchid flowers, and (c) qualitative analysis of rectal glands from male fruit flies attracted to the fruit fly orchid.

METHODS AND MATERIALS

Plants and Flowers. Orchid plants were originally collected from the Tenom Orchid Center, Tenom, Sabah, and from Pulau Kapas, W. Malaysia. Plants were grown in two localities in Penang—Tanjung Bungah and USM Campus. Flowers used for chemical analyses were covered with a clear plastic bag one day before blooming.

Collection of Fruit Flies. Fruit flies visiting the orchid flower were collected in a clear plastic bag, transferred into vials, and identified to their respective species. For rectal gland extraction, male flies were collected as they landed on a nylon stocking covering a mini wire cage over a fruit fly orchid flower. The rectal gland was removed under a dissecting microscope after cold immobilization. Each gland was homogenized in 20 μl of redistilled ethanol in a small V tube by using a fine glass rod. After centrifugation, a 1-μl aliquot was subjected to gas–liquid chromatographic and GC-MS analyses to qualitatively determine and confirm the presence of ME analogs in comparison with authentic compounds.

Pollinarium Removal by Fruit Fly. The process of pollinarium removal by a male *B. papayae* was observed in a large field cage ($2.1 \times 2.1 \times 2.1$ m^3). A plant bearing a bud was placed into the cage. On the day when the flower bloomed, a sexually mature male fruit fly was released into the cage for observation. The observation was repeated with another flower.

Identification of Pollinarium. Whenever possible, a fly bearing a pollinarium when found on or near an orchid flower was collected. The morphology and size of pollinarium on two flies were checked against those picked from *Bu. cheiri* and *Bu. patens* flowers, both under a dissecting microscope and scanning electron microscope (SEM). The dimensions of a single pollinarium stuck to the dorsum of a fly were measured from the SEM micrographs. To determine how long the

pollinia remained on the flies, two of the four wild flies caught bearing pollinaria were isolated and kept in a cage containing water and fly food sources, which were changed regularly, for observation.

Instruments. The volatile components were analyzed by GC-MS by using a HP 5989B mass spectrometer coupled with a HP 5890 series II plus gas chromatograph equipped with a HP-5 capillary column (30 m $\times$ 0.32 mm, 0.33-μm film thickness) programmed from 60° (2 min holding) to 240°C at a rate of 10°C/min, total ion monitor. Quantification of volatile chemicals was done on a HP 5790A gas chromatograph with the same program conditions as above by comparing the FID intensities with those of the standard sample of known concentrations using a HP 3396 integrator.

Extraction of Floral Fragrance. Flowers of *Bu. cheiri* were initially collected from Tenom, Sabah. Five flowers of *Bu. cheiri* were extracted with ethanol (ca. 10 ml/flower $\times$ 2). The combined ethanolic solution was filtered (Advantec filter paper No. 2), concentrated under reduced pressure ($\sim$20 mm Hg, below 30°C), and partitioned between an 8:1:1 mixture of hexane, benzene, and ethyl acetate (10 ml $\times$ 2) and water (5 ml). The organic layer was dried over anhydrous sodium sulfate and evaporated under partial vacuum to yield an oily material (26 mg), a portion of which was analyzed by GC-MS.

Headspace Analysis of Floral Fragrance. A flower of *Bu. cheiri* was wrapped with a small plastic bag with an air inlet and a small Porapak Q column outlet. A small stream of air was passed through the bag continuously for several hours. The column was changed every 2 hr and washed with dichloromethane (5 ml). The extract from each 2-hr period was concentrated and analyzed by GC.

Quantification of Fruit Fly Attractants. Quantification was performed for two separate flowers (sample 1: collected on August 28, 1996, one day after blooming without exposure to flies; and sample 2: collected on April 15, 1988, within 2 hr of blooming). Floral lip, column, petals, and medial and lateral sepals were separated from each *Bu. cheiri* flower. They were individually weighed, soaked in ethanol, and subjected to GC quantification.

Authentic Samples. Methyl eugenol (ME, 3,4-dimethoxyallylbenzene) was purchased from Tokyo Chemical Industries Co. Ltd. Pure (>96%) chemicals synthesized and used for comparison were 2-allyl-4,5-dimethoxyphenol, *trans*-coniferyl alcohol, and *Z*-3,4-dimethoxycinnamyl alcohol (Tan and Nishida, 1998).

RESULTS

Fruit Fly Species. When the flower first bloomed in the morning, many fruit flies were attracted to it (Figure 1A). At certain times in the morning, the flower was literally covered with fruit flies. They were generally seen lapping on the petals and sepals. The process of pollinarium removal and pollination was difficult to observe due to the arrangement of petals and sepals, which completely cover the column

cavity. A few flies were generally observed to visit and lap on the flower in late afternoon and evening; and all would leave just before sunset at ca. 19:30 hr. One of the flies attracted early in the morning was seen to be carrying the pollinarium (pollinium and viscidium) of the flower (Figure 1B). In captivity, the pollinarium remained stuck to the fruit fly (Figure 1C) for over a month until the fly died.

All visitors to the fruit fly orchid flower were male *Bactrocera* fruit flies. When the flower was covered with a screen, flies would land on the latter. The majority of the 276 flies collected belonged to *B. papayae*; while 1 *B. carambolae*, 17 *B. umbrosa*, and 2 individuals with intermediate characteristics between *B. carambolae* and *B. papayae* were identified. All species belonged to the ME-sensitive group of *Bactrocera* fruit flies.

Pollinarium Removal. Normally, a fly visiting a flower first lands on one of the lateral sepals, extends its proboscis, and begins lapping the surface. It eventually ends up on the lip. When it moves onto the see-saw lip, which is on a higher plane than the floral column, it laps and continues moving slowly along the lip. As the fly moves towards the column and nears the hinge, the lip suddenly tilts towards the column and, simultaneously, topples the fly head first into the column cavity. Presumably, the shallow groove and the basal flank of the lip ensure that the fly does not fall on its side. In the small enclosed space, the fly struggles to free and upright itself by moving backwards because it is squeezed between the closed lip and the floral column. As it retreats, it pushes the lip away from the column and at the same moment the thoracic dorsum touches the visidium and removes the pollinarium, which adheres dorsally to the fly's thorax (Figure 1C). The pollinia borne by wild *B. papayae* males showed identical characteristics to those removed from the fruit fly orchid. In captivity, two wild *B. papayae* males, bearing a pollinarium each, showed normal feeding and grooming activities in a cage. The pollinarium was not observed to fall from the flies.

Rectal Gland Components. Many of the male *B. papayae* flies attracted to the fruit fly orchid flower that was enclosed within a mini cage already possessed ME analogs. Chemical analysis of the rectal glands showed that 17 of the 22 flies had varying quantities of *trans*-coniferyl alcohol and 2-allyl-4,5-dimethoxyphenol. The identities of both compounds were confirmed through GC-MS analysis and comparison with authentic samples. These chemicals were not detected in the remaining five individuals ($<25\%$).

Comparison of Pollinarium. Each pollinarium has four pollinia—a larger outer pair that enclose a smaller inner pair of almost equal lengths. They differ in size and morphology from those removed from flowers of *Bu. patens*, in which each pollinarium had only one pair of pollinia. Figure 2 shows the SEM micrograph of pollinia of *Bu. cheiri* on the thoracic dorsum of a male *B. papayae.* The average dimensions (width $\times$ length $\times$ height) of pollinaria borne on native flies (two each) for *Bu. cheiri* and *Bu. patens* were $1.22 \times 1.27 \times 0.72$ mm and $0.92 \times 0.90 \times 1.52$ mm, respectively.

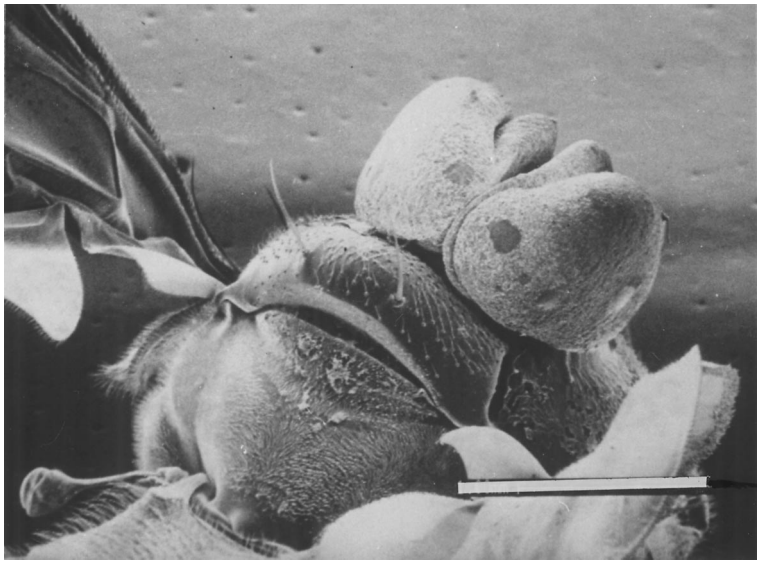


FIG. 2. Scanning electron micrograph of *Bulbophyllum cheiri* pollinarium on the thoracic dorsum of a fruit fly. Note: the four pollinia—two outer and larger pollinia enclosing a pair of smaller pollinia. Bar = 1.00 mm.

Chemical Analyses of Bu. cheiri Floral Scent. The major peak in the gas chromatogram of a *Bu. cheiri* floral extract had a retention time of 11.7 min and was identified as methyl eugenol (ME) (Figure 3) [MS: m/z(%) 178(100), 163(33), 151(9), 147(33), 107(22), 105(19), 103(19), 77(9)]. Also detected in individual

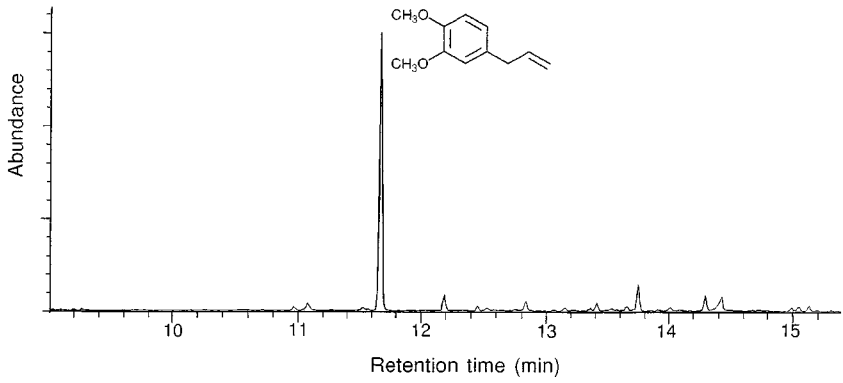


FIG. 3. Gas chromatogram of floral extract of a *Bulbophyllum cheiri* flower. The major peak is methyl eugenol.

TABLE 1. METHYL EUGENOL CONTENT IN INDIVIDUAL FLORAL PARTS OF TWO *Bulbophyllum cheiri* FLOWERS[a]

	Content (μg)		Fresh weight (mg)		Concentration (ppm)	
Floral part	A	B	A	B	A	B
Lateral sepals	310	35	413	321	750	107
Medial sepal	104	7	189	151	550	44
Petals	96	11	143	125	669	91
Lip	35	6	66	59	526	95
Column	40	6	174	139	230	41
Total	585	65	985	795	average 594	82

[a] Flower A was collected on April 15, 1998, within 2 hr after blooming; and flower B was collected on August 28, 1996, one day after blooming and not exposed to flies.

flowers were much smaller quantities of related phenylpropanoids such as eugenol (in one of the flowers), 1,2-dimethoxy-4-(1-propenyl)-benzene (retention time 12.8 min), a trimethoxypropenylbenzene (13.7 min), and a dimethoxypropenylphenol (14.3 min). The structure of the trimethoxypropenylbenzene could not be determined due to the small quantities available for further chemical analyses. The latter compound gave the same retention time and MS fragmentation pattern as 2-allyl-4,5-dimethoxyphenol, but its identity from other possible positional isomers has not been established due to the small amount of sample available.

The contents of ME in each floral part, given in Table 1, show that the lateral sepals contained the highest quantities. In the headspace analysis, ME was the major component in the first collection period (10:00–12:00 hr), declined in the second (12:00–14:00 hr), and was not detectable in the third 2-hr collection. Although the floral parts of a flower that has just bloomed contain much higher quantities of ME than those of a flower collected after one day of blooming, the latter contained larger quantities of dimethoxypropenylphenol.

DISCUSSION

The presence of ME analogs in the rectal glands of some flies attracted to the fruit fly orchid flower showed that they had previously fed on ME. Males of *B. papayae* that had fed on ME produced *trans*-coniferyl alcohol and 2-allyl-4,5-dimethoxyphenol, while those not exposed to ME did not (Nishida *et al*., 1988). These compounds are known to boost the pheromone and defense systems of *B. papayae* and *B. dorsalis*, thereby benefiting the male flies (Tan and Nishida, 1996).

After feeding on natural sources of ME, fruit flies did return to feed on ME released by the fruit fly orchid flower. When *B. papayae* males were exposed to a

high concentration of a pure ME source (isolated in a small-wire cage to prevent feeding) for over 1 hr in the laboratory, they did not respond to any subsequent ME source offered to them for one week. However, exposure for <1 hr did not cause this temporary habituation. Using a capture–mark–release–recapture technique and clear traps with an isolated ME source within a small-wire cage in the field, marked native *B. papayae* males were recaptured daily (Tan, 1985) or weekly (Tan and Serit, 1994) after being exposed to ME in an enclosed space for <1 hr. This ability of a ME-sensitive fruit fly to return repeatedly to a source of ME ensures its effectiveness and reliability as a pollinator.

Among the ME-sensitive fruit fly species, *B. papayae* and *B. umbrosa* are endemic in the forest, while *B. carambolae* is normally found in urban and cultivated areas. Sensitivity to ME varies from species to species. In the field, male *B. papayae* can be attracted to a spot containing ca. 1 ng of ME, but not *B. umbrosa* and *B. carambolae* (Tan, unpublished data). The relative content of ME in a freshly opened fruit fly orchid flower is high (>50% peak area) when compared with other orchid species: *Cattleya araguaiensis* Pabst, *Cattleya leopoldii* Lem., *Epidendrum nocturnum* Jacquin, *Gymnadenia conopea* (L.) R. Br., *Odontoglossum pulchellum* Lindl., *Oncidium sarcodes* Lindl., *Pescatorea dayana* Rchb.f., *Phalaenopsis violacea* Witte (Malaya type), *Rangaeris amaniensis* (Kraenzl.) Summerh., and *Zygopetalum crinitum* Lodd, which had ME from minor to 9% peak area of total floral volatiles (Kaiser, 1993). Using headspace–GC analysis, it was found that the fruit fly orchid flower releases ME into the environment at high levels during the morning, and this diminishes to undetectable quantities in the afternoon. Furthermore, ME contents were greatly reduced in the various floral parts when extracted a day after blooming. The high levels of ME account for the mild scent of *Bu. cheiri* flowers as opposed to it being "not scented" (Vermeulen, 1991), and to the high number of *B. papayae* males attracted to flowers during the morning hours.

It was suggested that ME-sensitive *Bactrocera* species are evolutionarily more advanced than RK-sensitive species (Tan, 2000a). The former consume and convert exogenous sources of ME to produce pheromonal and allomonal components, thus spending less metabolic energy, whereas the latter produce endogenous components and then sequester RK to boost the pheromonal and defense system (Tan, 2000b). Thus, the coevolution of *Bu. cheiri* and ME-sensitive *Bactrocera* species, especially *B. papayae*, has probably reached its peak. In this case, the flower does not only need to fully display its petals and sepals to ensure pollination, but it also possesses a similar defense system to that of the fruit fly through the production of ME analogs. The presence in the orchid flower of an allyldimethoxyphenol similar to that found in the rectal gland of most ME-sensitive fruit fly species is interesting. Male fruit flies use it as one of the components to attract other male fruit flies and to deter vertebrate predation (Nishida *et al.*, 1988; Tan and Nishida, 1998). Is this compound in the flower used primarily as an allomone to deter phytophagous vertebrate and, thus, protect the flower, i.e., to prevent unwelcome intruders from

damaging the precious floral reproductive organs, or is it one of the synomonal components? Without actual botanical and ecological evidence, the answer at this stage remains subjective.

An evolutionary trend from many to few pollinator species per plant species was observed in comparisons of ancestral versus recently derived monophyletic taxa in Orchidaceae (Tremblay, 1992). Further, reduction in the number of pollinator species per plant species is suggested to be a mechanism that may reduce the cost of pollen transfer. Taking this view, *Bu. patens*, with flowers that attract both the ME- and RK-sensitive *Bactrocera* species through zingerone, may be an ancestral species to *Bu. cheiri.* Therefore, floral synomone containing zingerone is probably the divergent point for *Bulbophyllum* species producing either RK or ME as attractants to lure fruit flies as efficient pollinators. As such, one would expect to find more species of wild orchids belonging to the genus *Bulbophyllum* that either produces RK or ME. A case in point is another *Bulbophyllum* species, *Bu. ecornotum*, which has a RK floral fragrance (Tan, 2000b). The flower of *Bu. ecornotum* is presently under going chemical analyses.

After attracting flies by the fragrance it releases, *Bulbophyllum* flowers ensure the removal and deposition of the pollinarium by its floral lip, which has been modified and adapted to a well-balanced structure of hinged see-saw lip that provides a floral mechanism to assist in pollination by flies. However, details of the fly's "temporary capture in a precise position" by the flower vary from one species of *Bulbophyllum* to another, depending largely on the design of the lip. In the fruit fly orchid flower, the adaxial end of its lip is broad, has high side-walls, and a shallow groove to ensure that a fruit fly always remains in an upright position when tipped head first into the column cavity. In this way, the pollinarium, when removed, is stuck on to the thoracic dorsum of the fly. In *Bu. patens*, the lip mechanism functions the same way in assisting pollination by tilting suddenly through an imbalance caused by the shifting of the fly's weight while feeding on the lip (Tan and Nishida, 2000). However, in *Bu. macranthum* the attracted fly (species not reported) slips from the glassy surface of the lateral sepal into the lip and then suddenly is tilted back, sending the fly abdomen-first into the column cavity. In this case, the pollinarium, when removed, sticks to the upper part of the fly's abdomen, and the fly, temporarily stuck to the floral stigma, extricates itself after releasing its grip on the lip (Ridley, 1890). Females of *Pholeomyia* spp. (unidentified), attracted by a fragrance of unknown identity, remove pollinarium via the movable lip mechanism by using wind assistance in *Bu. involutum*, *Bu. ipanemense* and *Bu. weddellii* (Borda and Semir, 1998b).

Hybridization has been observed between two sympatric *Bulbophyllum* species—*Bu. weddellii* and *Bu. involutum*, which have synchronous flowering and share the same species of pollinators in Brazil (Borba and Semir, 1998a). However, in Sabah, East Malaysia, hybridization between *Bu. cheiri* and *Bu. patens* is

unlikely—the former flowers in April–June, October, and December, and shares the same habitat in lowland forest at altitude 0–100 m with *Bu. patens*, which flowers in February and April (Vermeulen, 1991). While these two orchids are sympatric, with partially synchronous flowering (i.e., in April), and share a common ME-sensitive fruit fly species, *B. papayae*, as pollinator in the forest, hybridization through the transfer of pollinia by the ME-sensitive *Bactrocera* species will not occur for two reasons. First, the differences in pollinium size and shape may not allow for successful interspecific pollination; second, the ME-sensitive flies such as *B. papayae*, which are attracted at nanogram levels compared with zingerone at microgram levels, will be lured to the flowers of *Bu. cheiri* due to abundant ME released as a floral synomone (Tan and Nishida, 1998, 2000), leaving *Bu. patens* to attract only the RK-sensitive *Bactrocera* species found in the forest during the short overlap of flowering seasons in April.

In conclusion, this investigation shows a strong association between *Bu. cheiri* and male flies of the ME-sensitive *Bactrocera* species that results in mutual reproductive benefits through the release of ME in the floral fragrance. While the male fruit flies may find and consume ME from other plant sources to boost their pheromonal and allomonal systems, it is obvious that *Bu. cheiri* is entirely dependent on the male fruit flies for its pollination. Unfortunately, this unique association will be impacted by the current usage of ME in the management of pest fruit fly species in the agricultural sector. Therefore, the potential threat to the survival of *Bu. cheiri* in the rain forest must be seriously looked into before an eradication or suppression program for the ME-sensitive fruit fly species is implemented over a vast area.

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ALLELOPATHIC POTENTIAL AND CHEMICAL CONSTITUENTS OF VOLATILES FROM *Ageratum conyzoides* UNDER STRESS

CHUIHUA KONG,[1,2,*] FEI HU,[1] and XIAOHUA XU[2]

[1]*Institute of Tropical and Subtropical Ecology*
South China Agricultural University
Guangzhou 510642, China

[2]*State Key Laboratory of Elemento-Organic Chemistry*
Nankai University
Tianjin 300071, China

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Abstract—The allelopathic potential of *Ageratum conyzoides* was investigated under different environmental stress conditions, including nutrient deficiency, physical damage, 2,4-D treatment, competition with *Bidens pilosa*, infection with *Erysiphe cichoracearum*, and feeding by *Aphiids gossypii*. The inhibitory effects of *A. conyzoides* volatiles on peanut (*Arachis hypogaea*), redroot amaranth (*Amaranthus retroflexus*), cucumber (*Cucumis sativus*), and ryegrass (*Lolium multiforum*) increased when plants were grown under nutrient-deficient conditions or in competition with *B. pilosa*; however, there was no difference with physical damage or 2,4-D treatment. Phytoinhibitory effects decreased under fungal infection and aphid feeding. Volatiles from *A. conyzoides* plants infected with *E. cichoracearum* or exposed to *A. gossypii* feeding inhibited or killed fungi and insects. Precocenes and their derivatives, monoterpenes, and sesquiterpenes were the major volatile components of *A. conyzoides*.

Key Words—*Ageratum conyzoides*, allelopathy, environmental stress, volatile allelochemicals, chemical constituent, and phytochemistry.

INTRODUCTION

The allelopathic potential of many plants is intensified by exposure to various environmental stresses (Einhellig, 1987, 1996) and induces phytochemical variation (Josep and Joan, 1997; Kong et al., 2000). Several studies have shown that the

* To whom correspondence should be addressed. E-mail: chkong@scau.edu.cn

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amount of allelochemicals produced and released by plants is correlated to environmental factors (Dick, 1994; Loughhrin et al., 1995; Rose et al., 1995; Pare and Tumlinson, 1997; Agrawal, 1998). As a result, an increasing number of studies have focused on the relationship between environmental variation, plant chemical response, and the ecological function of plant allelochemicals.

Ageratum conyzoides is a perennial plant that grows in tropical and subtropical regions and is spread widely in natural and managed ecosystems. Its allelopathic potential has been demonstrated in previous studies (Kong et al., 1998a,b, 1999) and is achieved by releasing volatile allelochemicals into the environment (Kong et al., 1998a). Further research has shown that the allelopathic potential and major allelochemicals of *A. conyzoides* vary in different habitats, and that allelopathic synergism exists among *A. conyzoides* allelochemicals (Kong et al., 1998b, 1999). In this paper, the allelopathic potential and volatile chemicals from *A. conyzoides* were studied when plants were grown under various environmental stresses, including: nutrient deficiency, physical damage, treatment with 2,4-D, competition with *Bidens pilosa*, infection with the fungus *Erysiphe cichoracearum*, and feeding by the *Aphiids gossypii*.

METHODS AND MATERIALS

Culture of A. conyzoides. Plants were grown at the Ecological and Meteorological Experimental Station of South China Agricultural University (Guangzhou, China). Plots (4×5 m^2) were selected with two different fertility levels (Table 1). Fertility level 1 was regarded as containing sufficient nutrients to adequately support *A. conyzoides* growth (based on four years of observation and investigation), while fertility level 2 was considered nutrient deficient.

Black, ripe seeds of *A. conyzoides* were collected from uncultivated individuals growing at the experimental station during the summer of 1998. Seeds were dried in sunlight (48 hr) and stored in a sealed glass jar. In 1999, seeds were sown on different treatment plots and thinned to 600 plants/m^2 at the two-leaf stage. Sowing was done in October, and sampling of the above-ground portions of plants for chemical analysis was done in December.

Environmental Conditions. Except for conditions of nutrient deficiency, *A. conyzoides* was grown in fertility level 1 plots. Nutrient-deficient plants were

TABLE 1. ORGANIC MATTER AND SOIL FERTILITY

	Organic matter (g/kg)	Total N (g/kg)	Available N (mg/g)	Total P (g/kg)	Available P (mg/kg)	Total K (g/kg)	Available K (mg/kg)
Level 1	22.03	1.71	40.79	0.23	13.80	12.90	102.50
Level 2	6.12	0.29	10.88	0.49	0.15	6.06	22.01

grown in fertility level 2 soils. The above-ground portions of plants were collected at the peak flowering stage for chemical analysis.

A. conyzoides plants exposed to stresses other than nutrient deficiency were grown in fertility level 1 soils under nutrient-sufficient conditions as described below.

For physical damage, 50% of above-ground plant tissue was removed at the preflowering stage (60 days). Samples for chemical analysis were collected at the peak flowering stage (about 20–25 days after damage).

For 2,4-D stress, A solution of 2,4-D (250 μg/ml) was sprayed onto leaves and stems at the preflowering stage. The amount of spraying was 200 ml/m^2, half of the level used to control *A. conyzoides* in agricultural areas of South China. Plant samples for chemical analysis were collected at peak flowering stage (about 15–20 days after treatment).

For *B. pilosa* competition, seeds of *A. conyzoides* and *B. pilosa* were sown together on October 5. At the two-leaf stage, seedlings were thinned by hand to 300 plants/m^2 for each species. Samples for chemical analysis were collected at peak flowering stage as above.

For *E. cichoracearum* infection, *E. cichoracearum*-infected *A. conyzoides* plants were selected from the experimental station and planted into a plot containing healthy *A. conyzoides* at the preflowering stage. Most healthy plants showed visible signs of infection after 10 days. Plants for chemical analysis were collected at peak flowering stage.

For *A. gossypii* feeding, 1000 aphids were discharged evenly onto *A. conyzoides* plants at the beginning of the flowering stage. Plant samples for chemical analysis were collected after about 10 days when most leaves had been fed upon.

Collection of Volatiles. The above-ground portion of *A. conyzoides* (approx. 5.0 kg) was collected from each treatment plot on the sampling date. Samples were immediately placed into a vacuum desiccator equipped with a liquid nitrogen cold trap and pumped to 133 Pa. The oily volatiles were removed from the cold trap by injector after approx. 10 hr. Yields (volatile weight per plant, milligrams per gram fresh weight), bioassay, and chemical analyses were immediately determined.

Bioassays. Peanut (*Arachis hypogaea*), redroot amaranth (*Amaranthus retroflexus*), cucumber (*Cucumis sativus*), and ryegrass (*Lolium multiforum*) were used to bioassay for growth inhibition by *A. conyzoides* volatiles. Fungi included sheath blight (*Rhizoctonia solani*), cucumber gray mold (*Botrytis cinerea*), and Chinese cabbage sclerotinia rot (*Sclerotinia sclerotiorum*). The insects tested were red flour beetle (*Tribolium confusum*), Oriental armyworm (*Mythimna separata*), and northern mosquito (*Culex pipiens*). The biological actions on insects and fungi were evaluated by using the methods of Robertson and Preisler (1992). The inhibitory effect of volatiles on plant growth was evaluated following Kong et al. (1999). Uniform, germinated seedlings (30 seedlings with radicle lengths approx. 1 mm) from each of the bioassay plant species were placed into Petri dishes (10 cm diam.) containing filter paper and a thin layer of quartz sand in the bottom. The paper and

sand was moistened with Hoagland's nutrient solution (2.5 ml). The five uncovered dishes containing the various bioassay species were placed into a controlled environmental chamber (3 m^3) with a source of *A. conyzoides* volatiles. The rate of allelochemical volatilization was maintained at 200 mg/hr (monitored by gas chromatography) with proper air displacement pressure. Ordinary air served as the control for these experiments. Plants were grown for one week at $25 \pm 2^{\circ}$C under a 12L:12D photoperiod. Fresh weight, chlorophyll content, and peroxidase (POD) activity were determined for both treated and control plants. Chlorophyll content was determined using the technique developed by Baziramaberga et al. (1994) and POD activity was determined by the method of Hammerschmidt et al. (1982). All determinations were replicated three times under identical conditions.

Qualitative and Quantitative Analyses. Volatiles were analyzed by gas chromatography–mass spectrometry (GC–MS) on a Hewlett-Packard 5972 GC/MSD. Samples (1 μl) were injected onto an HP-5 bonded stage fused-silica capillary column (30 m $\times$ 2.5 mm) using the split mode. The initial oven temperature (70°C) was maintained for 2 min following injection. The temperature was then increased to 150°C at a rate of 20°C/min and then to 180°C at a rate of 10°C/min. After maintaining the column at 180°C for 5 min, the oven temperature was raised to 250°C at 15°C/min. Mass spectra were repetitively scanned from 35 to 450 amu every 2 sec. Ionization was elected in the electron impact mode (EI) at 70 eV. Volatiles were identified by peak matching against standards in the NIST 92 Computer Library or spectral similarity to an authentic reference compound (Aldrich Chemical Co.). The relative amounts of volatiles were calculated by integration of all peaks with areas greater than 0.1%.

Data Analyses. Impact effects were evaluated by the method of Williamson and Richardson (1988). The response index (RI) was determined as follows:

$$\text{If } T \geq C, \text{ then } RI = 1 - C/T; \qquad \text{If } T < C, \text{ then } RI = T/C - 1$$

where T is the treatment response and C is the control response. RI values range from $+1$ to -1, with positive values indicating stimulation and negative values indicating inhibition relative to controls. Absolute values of RI varied directly according to impact effects. A t test was used to analyze results of experiments evaluating impact effects of volatiles on plants, fungi and insects.

Quantification of the volatile chemical components was obtained from independent GC-MS measurements. Data are presented as means $\pm$ SEM. ANOVA with Duncan's multiple-range test was used to analyze the data.

RESULTS

Allelopathic Potential under Stress. Volatiles from *A conyzoides* inhibited the growth and development of peanut, redroot amaranth, ryegrass, and cucumber (Figure 1). Inhibition of plant growth was significantly different under nutrient

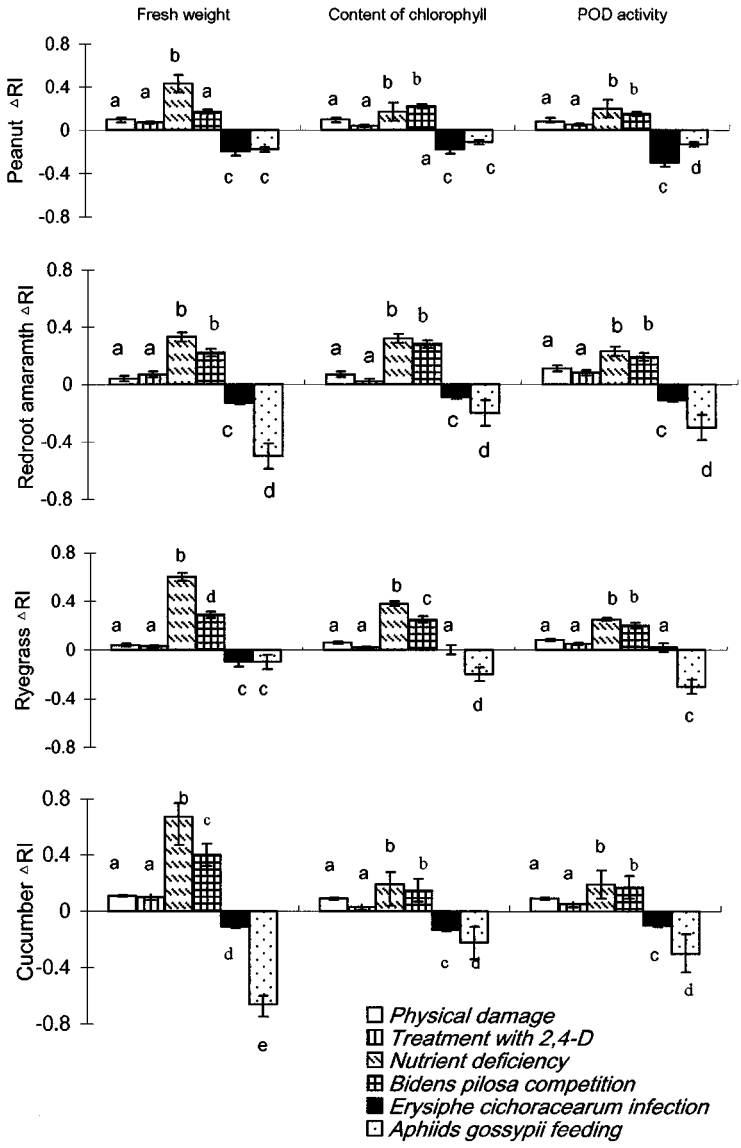


FIG. 1. Inhibitory effects of the volatiles from *A. conyzoides* on plants tested with different stress factors. ($^{\triangle}$RI = control RI—treatment RI, control was nutrient sufficiency.)

deficiency and *B. pilosa* competition. Significant differences in allelopathic potential were not observed in response to nutrient sufficiency (control), physical damage, or treatment with 2,4-D. The allelopathic potential of *A. conyzoides* was enhanced under conditions of nutrient deficiency and *B. pilosa* competition.

The absolute amounts of volatiles from *A. conyzoides* increased greatly while the inhibitory effects of these chemicals on plant growth decreased dramatically under *E. cichoracearum* infection and aphid feeding by *A. gossypii*. It is possible that allelochemicals produced in response to *E. cichoracearum* infection and *A. gossypii* feeding not only inhibit the growth of neighboring plants, but also defend plants against microbial infection and insect feeding. To test this, some common insects and fungi were exposed to volatiles produced by *A. conyzoides* either infected with *E. cichoracearum* or subjected to aphid feeding by *A. gossypii*. The volatiles had significant inhibitory or killing effects on the insects and fungi tested (Figure 2). Volatiles produced by *A. conyzoides* in response to infection by *E. cichoracearum* inhibited the growth of *Rhizoctonia solani*, *Botrytis cinerea*, and *Sclerotinia sclerotiorum*. Similarly, *A. conyzoides* exposed to *A. gossypii* produced volatiles that killed *Tribolium confusum* (adult), *Mythimna separata* (the third larva), and *Culex pipiens* (the fourth larva). However, purified precocene II, the major component of *A. conyzoides* volatiles in both control and stressed plants, was less effective at inhibiting the growth of fungi or killing of insects. Obviously, There may be other allelochemicals with antifungal and antiinsect activity produced by *A. conyzoides* infected by *E. cichoracearum* or fed on by *A. gossypii*.

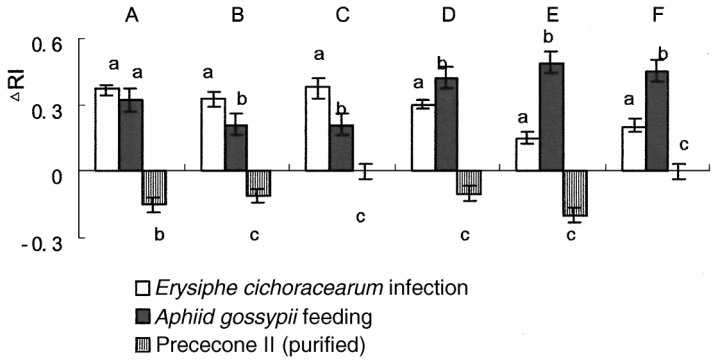


FIG. 2. Inhibitory and killing effects on tested insects and fungi of the volatiles from *Ageratum conyzoides* growing under *Erysiphe cichoracearum* infection and *Aphiid gossypii* feeding. (A: *Rhizoconia solani* B: *Botrytis cinerea* C: *Sclerotininia sclerotiorum* D: *Tribolium confusum* (adult) E: *Mythimna separata* (the third larve, Compared to control, all treatments delayed molting 24 h) F: *Culex pipiens* (the forth larve). The treatment concentrations to *M. separata* were 1000 μg.g^{-1}, and to *C. pipiensis* were 100 μg.g^{-1}, the others were 500 μg.g^{-1}. other remarks were the same as Fig. 1 footnote.)

Impact of Environmental Stress on Volatile Constituents. When exposed to different environmental stresses, *A. conyzoides* demonstrated different biological actions as demonstrated above. These actions were correlated with varying volatile compositions. Qualitative and quantitative analysis of volatile constituents was accomplished by GC-MS and showed significant variation in response to the various stress factors (Table 2). Precocenes and their derivatives (2H-benzopyran), monoterpenes, and sesquiterpenes were the main constituents. Control plants, those subjected to physical damage and treatment with 2,4-D, showed weak allelopathic potential and were characterized by precocene I and II levels of around 57 and 37 mg/g fresh wt, respectively; β-caryophylene levels of control plants were approximately 26 mg/g fresh wt. Under nutrient-deficient conditions and/or *B. pilosa* competition, *A. conyzoides* elicited a stronger alleopathic response and produced significantly higher levels of both precocenes and β-caryophllene. Precocene levels decreased in response to *E. cichoracearum* infection (i.e., precocene I) and aphid feeding (precocene II), while β-caryophllene levels did not change significantly. The levels of various mono and sesquiterpenes significantly increased in response to these stresses. These differences resulted in differential allelopathic effects and biological actions on insects and fungi.

DISCUSSION

There were significant differences in plant growth inhibition and biological actions on the fungus and aphids tested in response to phytochemical changes in *A. conyzoides* exposed to various biotic and abiotic stresses. It is unclear how *A. conyzoides* may release these volatile allelochemicals into the environment; however, this research documents that *A. conyzoides* can produce more and different volatile allelochemicals under adverse conditions—particularly under nutrient deficiency, competition with *B. pilosa*, infection with *E. cichoracearum*, and aphid feeding by *A. gossypii*. Physical damage and treatment with 2,4-D did not affect the allelopathic potential of *A. conyzoides* or the concentration of the major compounds present, i.e., anthropogenic factors did not seem to induce *A. conyzoides* to produce higher levels of allelopathic phytochemicals.

Allelopathic capability could be an adaptive mechanism and strengthen the ability to compete with other plants under adverse conditions (Tang, 1995). Although the allelopathic potential of *A. conyzoides* was related to environmental stress, not all stress factors intensified the plants' allelopathic potential. Only a few natural environmental stress factors induced and/or regulated the production of allelochemicals and caused changes in the qualitative and quantitative characteristics of the phytochemical constituents. The main ecological functions of volatiles from *A. conyzoides* infected with *E. cichoracearum* or exposed to aphid feeding may not be for inhibition of neighboring plant growth; infection and aphid feeding

TABLE 2. QUALITATIVE AND QUANTITATIVE ANALYSIS OF VOLATILES FROM *A. conyzoides* UNDER DIFFERENT STRESS CONDITIONS[a]

Chemical constituents	Nutrient sufficient (control)	Physical damage	Treatment with 2,4-D	Nutrient deficient	*B. pilosa* competition	*E. cichoracearu* infection	*A. gossypii* feeding
Precocene II	58.09 ± 4.43a[b]	57.99 ± 4.43a	57.81 ± 4.80a	176.48 ± 24.33b	82.05 ± 3.74c	53.23 ± 6.82a	52.41 ± 3.72b
Precocene I	38.37 ± 3.81a	34.46 ± 2.59a	37.02 ± 3.44a	71.82 ± 7.35b	55.86 ± 2.55c	22.66 ± 2.59d	32.16 ± 4.22a
β-Caryophllene	26.39 ± 3.02a	26.67 ± 2.12a	24.63 ± 2.12a	39.73 ± 2.42b	37.34 ± 1.70b	22.56 ± 2.53a	21.83 ± 2.00a
α-Bisabolene	4.81 ± 0.47a	3.80 ± 0.32a	4.04 ± 0.31a	5.10 ± 0.31a	6.01 ± 0.24b	13.43 ± 3.39c	17.39 ± 0.96d
α-Farnesene	2.09 ± 0.21a	2.18 ± 0.37a	2.45 ± 0.21a	2.37 ± 0.11a	2.06 ± 0.10a	8.91 ± 1.11b	19.06 ± 0.53c
β-Farnesene	1.77 ± 0.15a	2.09 ± 0.21a	2.51 ± 0.22a	2.02 ± 0.10a	1.60 ± 0.08a	14.89 ± 0.56b	12.32 ± 2.10c
Germacrene	1.35 ± 0.12a	1.74 ± 0.17a	0.72 ± 0.07b	1.12 ± 0.22a	1.36 ± 0.07a	11.39 ± 0.16c	12.10 ± 0.26c
α-Cubebene	0.95 ± 0.08a	0.94 ± 0.07a	1.40 ± 0.11a	0.78 ± 0.05a	0.90 ± 0.18a	9.85 ± 0.78b	7.11 ± 0.70c
Eupatoriochromene	0.78 ± 0.23a	1.23 ± 0.10b	0.79 ± 0.06a	1.83 ± 0.10b	1.62 ± 0.08b	4.61 ± 0.30c	5.74 ± 0.98c
Fenchyl acetate	0.76 ± 0.02a	1.66 ± 0.07b	0.64 ± 0.05a	0.45 ± 0.08a	0.64 ± 0.05a	2.31 ± 0.15b	5.25 ± 0.55c
Sesquiterpene	0.64 ± 0.12a	0.77 ± 0.11a	0.86 ± 0.08a	2.35 ± 0.46b	1.77 ± 0.11c	2.25 ± 0.26d	4.44 ± 0.59e
Hydrocarbon nerol	0.59 ± 0.10a	1.09 ± 0.08b	1.10 ± 0.32b	1.55 ± 0.09b	1.11 ± 0.16b	0b	1.86 ± 0.34b
Caryophllene oxide	0.55 ± 0.05a	0.84 ± 0.08b	0.83 ± 0.07b	0.49 ± 0.10a	0.60 ± 0.06a	3.34 ± 0.39c	2.01 ± 0.47c
Bornyl formate	0a	0a	0a	0a	0a	0a	7.25 ± 1.24b
Nerolidal	0.52 ± 0.03a	0b	0.68 ± 0.10a	0.52 ± 0.10a	0b	2.89 ± 0.10c	1.23 ± 0.09d
β-gurijunene	0.44 ± 0.10a	0b	0b	0.90 ± 0.18c	0.92 ± 0.11c	1.91 ± 0.11d	1.39 ± 0.16d
Camphene	0.39 ± 0.18a	0b	0b	0.68 ± 0.07c	0.44 ± 0.03a	1.60 ± 0.05d	2.38 ± 0.97e
Copaene	0.35 ± 0.10a	0b	0b	0.75 ± 0.11b	0.68 ± 0.08b	1.75 ± 0.71c	2.44 ± 0.98d
β-elemene	0a	0a	0a	0a	0.83 ± 0.07b	0.98 ± 0.17b	1.39 ± 0.16c
Methanazulene[c]	0a	0a	0a	0a	0a	1.53 ± 0.72b	1.68 ± 0.18c
Tricycoundecane[d]	0a	0	0a	0a	0a	1.49 ± 0.02b	0a
α-Muurolene	0a	0a	0a	0a	0a	0.44 ± 0.08b	0a
Demethoxyencecalin	0a	0a	0a	0a	0a	0a	2.10 ± 0.36b
Audroenececalinol	0a	0a	0a	0a	0a	0a	2.09 ± 0.19b
Total	138.84 ± 13.22a	134.74 ± 10.72a	135.48 ± 11.96a	308.94 ± 36.18b	195.79 ± 9.41c	209.02 ± 21.00c	215.63 ± 21.75c

[a] Values expressed as mean ± SEM (mg/g × 100 fresh wt).
[b] Values in a row not followed by the same letter are significantly different, $P = 0.05$, ANVOA with Duncan's multiple-range test.
[c] Decahydro-4,8,8-trimethyl-9-methylene-1,4-methanoazulene.
[d] 2,6,6,9-Tetrametnyltricyclo[5,4,0,$O^{2.8}$]undecane-9.

were more harmful to *A. conyzoides* than competition with neighboring plants. In fact, many kinds of allelochemicals not only interfere with neighboring plant growth, but also affect the behavior and development of insects and fungi. Allelochemicals have multiple ecological functions (Seigler and Price, 1976; Saiki and Yoneda, 1981; Berenbaum, 1985; Romeo, 1998). They may serve as antiherbivore, antifungal, and/or antibacterial compounds, phytotoxins, attractants, internal regulators of growth and development, seed germination stimulants, and inhibitors, etc. Our results suggest that the allelopathic potential of *A. conyzoides* is correlated with its growth conditions, and the categories and amounts of volatile allelochemicals from *A. conyzoides* could be induced by a few of these natural stress factors.

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MICROBE INHIBITION BY *Tribolium* FLOUR BEETLES VARIES WITH BEETLE SPECIES, STRAIN, SEX, AND MICROBE GROUP

HOLLY R. PRENDEVILLE and LORI STEVENS*

Department of Biology
University of Vermont
Burlington, Vermont 05405

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Abstract—*Tribolium* flour beetles produce defensive compounds, including quinones, putatively aimed at deterring predators and inhibiting microbes. Here we examine how effective the defensive secretions of *Tribolium confusum* and *T. castaneum* are at inhibiting growth of various microbes and how this varies with species, geographic strain, and sex of the beetles. We explore differences at both the kingdom and species level of common flour microbes in their susceptibility to defensive compounds. Beetle species and strains vary in their ability to inhibit microbial growth. In addition, microbes vary in their sensitivity to the beetles' defense compounds. The capability to suppress microbial growth is likely under stabilizing selection with optimum quinone production varying among populations and may be dependent on several environmental factors including temperature, humidity, and predators.

Key Words—*Tribolium confusum*, *Tribolium castaneum*, microbe inhibtion, defense compounds, benzoquinones, Coleoptera, Tenebrionidae, beetle-microbe interactions.

INTRODUCTION

Flour beetles, genus *Tribolium*, are an agricultural pest commonly found in flour silos (Sokoloff, 1974). Their habitat is rich in microbial competitors such as bacteria, yeast, and fungi (Abramson et al., 1980; Borjesson et al., 1989; Yezerski et al., 2000). Presumably, to both inhibit microbe growth and deter predators (Dettner, 1993; Eisner et al., 1998), *Tribolium* produce and store defensive secretions in the form of a foul-smelling yellow fluid. The secretion of *T. confusum* consists of

* To whom correspondence should be addressed. E-mail: Lori.Stevens@uvm.edu

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ethyl-1,4-benzoquinone (EBQ) and methyl-1,4-benzoquinone (MBQ); while that of *T. castaneum* contains EBQ, MBQ, and small quantities (<1%) of 2-methoxy-1,4-benzoquinone (Pappas and Morrison, 1995; Unruh et al., 1998; Yezerski et al., 2000). Benzoquinones are toxic and nonspecific elements of chemical defense in many insects (Blum, 1981). In excess, these benzoquinones turn the beetles' flour medium pink and cause developmental abnormalities in larvae and pupae (Chapman, 1926; Happ, 1968; Sokoloff, 1974, 1977). Previous studies have shown that benzoquinones inhibit the growth of yeast, bacteria, and fungi (Söderhall and Ajaxon, 1982; Nishina and Uchibori, 1991), in addition to deterring other insects and predators (Dettner, 1993; Eisner et al., 1998).

Benzoquinones are produced only in adult beetles, from a pair of prothoracic and postabdominal glands that are reservoirs for benzoquinones [for morphological description of *T. confusum* see Happ (1968) and for *T. castaneum*, see Roth and Stay, (1958)]. An individual beetle can be characterized by its internal, or glandular, amount and the external amount that reflects the nonvolatized residue from excretion. Internal and external quinone quantities are known to vary among species, geographic strains within species, and sexes (Pappas and Morrison, 1995; Unruh et al., 1998; Yezerski et al., 2000). The genetic basis of this variation has been examined for *T. confusum*; internal amounts of benzoquinones are genetically variable, whereas external amounts are largely determined by nongenetic factors (Yezerski, 1990).

In this paper, we examine the efficacy of compounds produced by *Tribolium* flour beetles to inhibit the growth of microorganisms that commonly occur in flour. We investigate whether the zone of inhibition from an individual beetle varies among geographic strains within a species and between the sexes. We also investigate whether microbes vary in their susceptiblity to the beetles' defensive secretions.

METHODS AND MATERIALS

Beetles. To investigate intra- and interspecific variation, we examined *T. confusum* strains b-+ and b-Pak, and *T. castaneum* strains c-sm and c-+ (see Yan and Norman, 1995, for description). Beetles were raised in stock jars with 100 g of single-sifted standardized (SSS) flour medium per 200 adults. The flour medium, SSS, is fine, sifted whole-wheat flour, sterilized by heating to 55°C for at least 24 hr, and supplemented with 5% dry weight, powdered brewer's yeast. Beetle colonies were maintained at 70% relative humidity and 29°C in dark incubators. Individuals were sexed as pupae, and individually placed into 1-dram vials containing 1 g SSS. Beetles were assayed at 31–52 days after eclosion.

Microbes. We examined effects of four yeast species [*Rhodotorula glutinis* (RG), *Saccharomyces microellipsoides* (SM), *Pichia anomala* (PA), *Cryptococcus albidus* (CA)]; and two bacteria species [*Bacillus subtillis* (BS) and *Bacillus*

licheniformis (BL)]. Microbe species were obtained from the USDA Agricultural Research Service Culture Collection (http://www.ars.usda.gov/is/np/systematics/contents.htm). We cultivated microbe cultures from lyophilized preparations by suspending pellets in 10- × 75-mm tubes with 0.5–0.7 ml of liquid yeast medium (YM). The YM agar was prepared according to a protocol outlined by the USDA: 3 g of yeast extract, 3 g of malt extract, 5 g of peptone, 10 g of glucose, 1 liter of distilled water, and 20 g of agar. Cultures were initially grown for three weeks in 15-ml Corning centrifuge tubes with 10 ml of YM broth at ambient temperature, approximately 26°C. We refreshed microbe suspensions every two to three weeks by adding 1–2 ml of culture solution to 10 ml of fresh YM broth. After addition of YM broth, cultures were allowed to grow for a minimum of three days prior to plating. We transferred all microbe suspensions with filtered pipet tips to minimize contamination.

Microbe lawns were grown on YM agar in Petri dishes to test for their sensitivity to compounds. Yeast medium agar was poured into 100-mm plates, allowed to congeal overnight, and plates were either used the next day or refrigerated until needed to reduce the risk of contamination. Plates were inoculated by the addition of 0.8–0.9 ml of one of the six microbe suspensions. The plate was immediately swirled to ensure even microbe lawn growth, then the suspension was allowed to absorb into the plate for 10–20 min.

Assay. To determine an assay for microbe inhibition from a single beetle, pilot assays investigated potential techniques that would cause excretion of compounds in the prothoracic and postabdominal glands. The pilot assays included: (1) grinding a beetle onto a filter paper disk with a glass rod and placing the disk on an inoculated plate; (2) placing a live beetle upside down on the agar (beetles often cannot turn themselves over; therefore, the effect is localized); (3) freezing a beetle for 1 hr at −4°C and then placing it on a Petri dish; and (4) placing a beetle frozen at −80°C for 5–15 min on an inoculated plate. Freezing triggered release of a yellow substance from the thorax and abdomen, at the location of the glands that produce quinones. The −80°C freezing protocol was chosen because it was easy, consistent, and did not involve a solvent. Inoculated plates with previously frozen beetles were placed in a dark drawer at ambient temperature for 24 hr, after which we measured the area of inhibition. A zone of inhibition is the area around a beetle where a microbe lawn or colony is not visible to the unaided eye. The zone was measured by placing a Petri dish on a 1-mm-square sheet of graph paper and counting the number of squares. Thus, the assay represents the zone of inhibition for a single beetle and includes the total (internal + external) defensive secretions produced by that beetle.

We quantitatively assayed five of the six microbes with a total of 401 beetles; however, we only assayed RG with b-+ and were unable to assay b-Pak with BL because of contamination. Sample sizes for each microbe–beetle combination ranged from 16 to 28, with a mean of 21.

Statistical Analysis. Area of inhibition was square-root-transformed prior to statistical analysis to achieve a normal distribution of the values. Analysis of variance was carried out with the statistical program JMP (SAS Institute, 1995) on a Macintosh computer. The analysis is a nested ANOVA. Beetle strains are nested within species, and microbe species nest into bacteria and yeast groups, and the design is unbalanced. Thus, orthogonal contrasts were used to test for main effects and interactions. Contrasts were generated a priori to allow us to investigate the effect of beetle sex, species, and strain in addition to microbe group with a controlled experiment-wise error rate.

RESULTS

All beetle strains examined inhibited microbial growth, and all microbe species were sensitive to beetle secretions (Figure 1, Table 1). A zone of inhibition

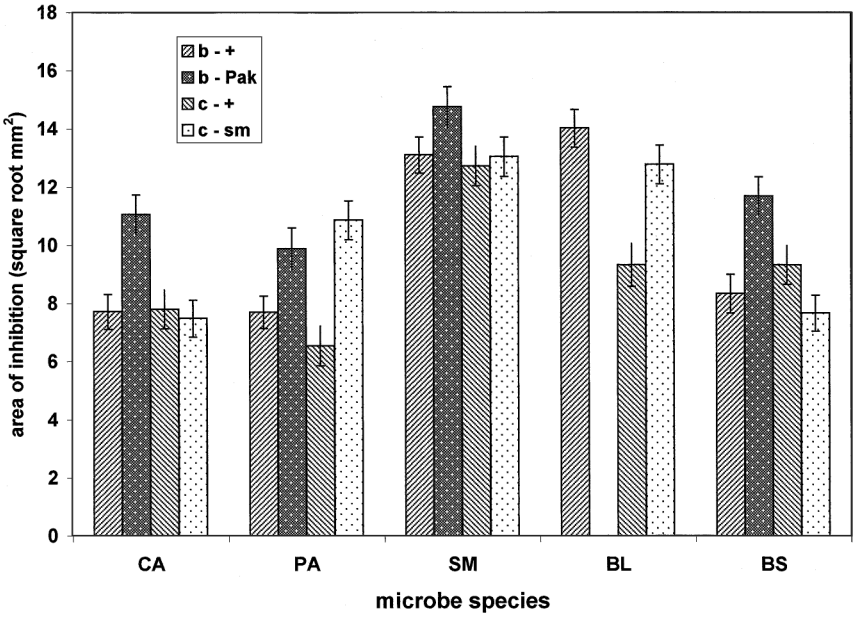


FIG. 1. Area of inhibition (square root transformation of mm^2) of microbe growth for two genetic strains of *Tribolium confusum* (b-+ and b-Pak) and two of *T. castaneum* (c-sm and c-+). The origin of the beetle strains is described in the text. Microbes from two kingdoms, yeast and bacteria, were examined. Results are presented for three species of yeast (*Cryptococcus albidus*, CA; *Pichia anomala*, PA and *Saccharomyces microellipsoides*, SM) and two of bacteria (*Bacillus licheniformis*, BL, and *B. subtillis*, BS).

TABLE 1. ANOVA OF TRANSFORMED AREA OF INHIBITION[a]

Main effects	SS	*F* ratio	*P*
1. Sex of beetle	50.44	2.47	0.010
2. Species of beetle: *T. confusum* vs *T. castaneum*	79.98	3.11	0.002
a. Strain of *T. confusum*: b+ vs bPak	316.59	6.20	<0.001
b. Strain of *T. castaneum*: c+ vs cSM	71.39	2.94	0.004
3. Microbe group: bacteria vs. yeast	53.97	2.56	0.010
a. Species of bacteria: BL vs. BS	383.29	6.82	<0.001
b. Species of yeast			
1. CA vs. PA, SM	351.96	6.53	<0.001
2. PA vs. SM	800.39	9.85	<0.001
Interactions: 1 × 3: Sex × microbe group	39.11	2.18	0.030
2a × 3: Strain of *T. confusum* × microbe group	50.44	2.47	0.010
2b × 3: Strain of *T. castaneum* × microbe group	88.64	3.28	0.001
2b × 3a: Strain of *T. castaneum* × species of bacteria	126.85	3.92	<0.001
2b × 3b1: Strain of *T. castaneum* × species of yeast: 1.	78.41	3.08	0.002
2b × 3b2: Strain of *T. castaneum* × species of yeast: 2.	74.42	3.00	0.003
1 × 2 × 3b2: Sex × beetle species × species of yeast: 2.	32.16	1.97	0.050
1 × 2a × 3: Sex × Strain of *T. confusum* × microbe group	62.43	2.75	0.006

[a] Orthogonal contrasts examined main effects and interactions, significant interactions ($P < 0.05$) are shown.

was visible around the beetles even weeks after the beetles were placed onto Petri dishes. The main effects from the ANOVA are shown (Table 1), along with the statistically significant interactions ($P < 0.05$). Beetle sex, species, and strain significantly affected the area of inhibition, as did the microbe group and strain (Table 1). The mean zone of inhibition from female beetles was larger than from males. On average, *T. confusum* had a larger zone of inhibition than *T. castaneum*. This is because strain b-Pak has such high values rather than that *T. confusum* has an overall greater zone of inhibition (Figure 1). Both sets of strains within beetle species were significant, suggesting a wide potential range of adaptation within species. On average, the zone of inhibition for bacteria was larger than for yeast (main effect 3: bacteria vs, yeast, $F = 9.85$, $P < 0.01$); however, the suppression of the yeast species SM was significantly greater than one of the two bacteria species tested. Thus, microbe species vary in their sensitivity to compounds produced by *Tribolium* beetles, but the pattern of sensitivity does not reflect phylogeny at the yeast and bacteria level.

Of the 27 interaction terms, eight were significant ($P < 0.05$) (Table 1). These are a priori tests, based on the hierarchical nesting of the groups examined, so multiple comparisons are controlled for. Four of the eight interactions include the strain of *T. castaneum* (2b, Table 1), four include the microbe group (3, Table 1), and three include sex (1, Table 1). The interactions indicate when an effect has a different order or magnitude. For example, the strain of *T. castaneum* (c-+ vs

c-sm) × species of bacteria (BL vs BS) interactions indicates that the zone of inhibition of c-+ relative to c-sm is significantly different for the two bacteria species. Overall, the statistical analysis reveals that the microbes are inhibited by the beetles and that several factors affect the level of inhibition; however, the taxonomy or phylogeny of the groups examined does not control the level of inhibition.

DISCUSSION

Tribolium beetles produce compounds that inhibit the growth of microbes commonly found in their flour medium (Figure 1, Table 1). Biochemical analysis has detected only benzoquinones in these secretions; however, our assay tested the ability of whole beetles to inhibit microbial growth rather than explicitly testing the quinones. Beetles vary both between species and between genetic strains within species in their capability to suppress microbe growth. Microbes vary at the kingdom and species level in their sensitivity to the beetle's defense compounds.

Tribolium confusum and *T. castaneum* are believed to have evolved as inhabitants of bark, decomposing plants, food caches of Hymenoptera, and rodent nests (Sokoloff, 1974). Present populations of *Tribolium* are cosmopolitan and commonly found in flour mills and grain silos. In these habitats, *Tribolium* encounter and consume microorganisms (Thatcher et al., 1953) and, in so doing, increase their fitness (Sinha, 1966; Wicklow and Stevens, 1997). However, high benzoquinone quantities cause abnormal development in larvae and pupae, as well as death in adults (Chapman, 1926; Happ, 1968; Sokoloff, 1974, 1977). Beetles are attracted to flour containing fungi and bacteria (Van Wyk et al., 1959) and are deterred from flour with high quantities of quinones (Loconti and Roth, 1953).

The variation in the ability to inhibit microbe growth suggests that quantities of defense secretions result from stabilizing selection combined with environmental factors at the individual or population level. Populations in different environments may be selected for distinct quinone levels if microbe growth or predators vary among environments. Temperature and humidity may affect microbe growth and, thus, climate may indirectly affect quinone levels. Stabilizing selection may occur for this trait with a beetle strain finding a balance between permitting the growth of beneficial microbes, controlling harmful microbes, deterring predators, and reducing harm to the beetle itself.

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PARTHENOGENESIS, CALLING BEHAVIOR, AND INSECT-RELEASED VOLATILES OF LEAFMINER MOTH *Phyllonorycter emberizaepenella*

RAIMONDAS MOZŪRAITIS,[1*,2] VINCAS BŪDA,[1] ILME LIBLIKAS,[2] CARL RIKARD UNELIUS,[2] and ANNA-KARIN BORG-KARLSON[2]

[1]*Laboratory of Chemical Ecology*
Institute of Ecology
Akademijos 2, LT-2600 Vilnius, Lithuania

[2]*Department of Chemistry, Organic Chemistry*
Royal Institute of Technology
SE-100 44 Stockholm, Sweden

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Abstract—We proved that the leafminer moth *Phyllonorycter emberizaepenella* (Lepidoptera: Gracillariidae) reproduces by parthenogenesis of the thelytoky type. Despite a complete absence of males, parthenogenetically reproducing females diurnally demonstrated the calling posture normally used for releasing signaling compounds. Two compounds, which we collected from a calling female, were identified as potential sex pheromone components: (8*E*,10*E*)-8,10-tetradecadienyl acetate and (8*E*,10*E*)-8,10-tetradecadienol, the latter occurring only in trace amounts. In field experiments, no males were attracted to traps baited with either the potential sex pheromone or with virgin females. Both the pattern of behavior and the chemical characteristics of the pheromone of *Ph. emberizaepenella* species were similar to those known for Lepidoptera with the usual amphimictic mode of reproduction. Theoretical speculations that in thelytoky, where there is no need to find a sexual partner, the individuals would obtain certain advantages due to reduction in their sexual behavior, were, thus, not confirmed for *Ph. emberizaepenella*.

Key Words—Thelytoky, diurnal rhythm, communication behaviour, (8*E*,10*E*)-8,10-tetradecadienyl acetate, (8*E*,10*E*)-8,10-tetradecadienol, Lepidoptera, Gracillariidae.

* To whom correspondence should be addressed. E-mail: Raimozu@julius.ktl.mii.lt

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INTRODUCTION

The parthenogenetic mode of reproduction in animals has a number of drawbacks and advantages when compared with amphimixis. One potential advantage is the absence of mate finding (Gilyarov, 1982; Went, 1984). However, from the viewpoint of the theory of optimality (Rozen, 1969), for altering the genetic program (including that of sex pheromone production and calling behavior), certain energy expenses are needed, and these are justified only when there will be a benefit greater than the cost of carrying out these modifications. With this in mind, the detailed investigation of both chemical and behavioral elements related to "searching" for a sexual partner in parthenogenetically reproducing animals is of interest.

Insects are convenient objects for such studies, especially species that use chemical communication while locating their mates. The aim of this study was to verify the parthenogenetic reproduction of the widespread leafminer moth *Phyllonorycter emberizaepenella* that was reported earlier (Būda et al., 1991) but never confirmed, as well as to elucidate the sexual behavior of this moth and to determind whether it releases potential sex pheromones during the calling.

METHODS AND MATERIALS

Insects

Common honeysuckle shrubs, *Lonicera xylosteum* L. leaves with *Ph. emberizaepenella* (Z.) (Lepidoptera, Gracillariidae) mines were collected at the end of October 1989 and 1996–1998 and held in an outdoor container on moistened peat moss until the following spring. Specimens were collected from Vilnius (Žemieji Paneriai) and from four other localities in Lithuania. The maximum distance between collection sites was 300 km. In addition, nondiapausing individuals were collected at the beginning of June 1999. The leaves with pupae were placed in 28-ml glass vials, three mines per vial, and held under an artificial 16L:8D photoperiod. A daylight lamp of 1000 W (type DRF, for greenhouses) was used. The temperature was maintained nearly constant during the scotophase, with irregular fluctuations within the range $14 \pm 1^{\circ}$C. Within 2 hr of the start of the photophase, it was gradually raised to $20 \pm 1^{\circ}$C, kept constant until 2 hr before the end of the photophase, and then gradually decreased, reaching 14°C when the next scotophase began. Following emergence, the adults were collected daily just after the onset of the photophase, then sexed and placed into individual holding vials (28-ml volume), and provided with a solution of 5% of sucrose in water.

Parthenogenetic Mode of Reproduction

Three 2-day-old virgin females of *Ph. emberizaepenella* were placed in individual containers together with green branches of the honeysuckle and were left to

oviposit for one day. The branches were steeped in Knop's solution (Grodzinskii and Grodzinskii, 1973). This food source was sufficient for the complete development of the progeny.

Diurnal Rhythm of Female Calling Behavior

At emergence, 32 virgin females were placed in individual glass vials and their behavior was observed over a four-day period. During experiments, females were provided with a solution of 5% of sucrose in water. Observations were made every 30 min during the last 2 hr of the scotophase until the 8th hour in the photophase, and hourly during the remaining period of a photocycle. Observations during the scotophase were made using a 4.5-V red lamp light. Females were classified as calling when the abdomen was raised clearly above the wings (Figure 1B).

Collection of Compounds

Compounds released from calling females were collected using the solid phase microextraction (SPME) technique (Borg-Karlson and Mozuraitis, 1996). The SPME fiber (100-mm polydimethylsiloxane) was purified at 225°C for about 10 min in a GC injector before being placed a few millimeters away from the abdominal tip of a calling female for 2–4 hr at the beginning of the calling period. Then the SPME fiber was inserted in a glass tube (1.75-ml volume), containing undecyl acetate (10 ng) and pentadecyl acetate (100 ng) on the inner wall as internal standards, and the sorption continued for another 15 min at 22°C. The volatiles from 12 females were collected individually during their calling and noncalling periods and analyzed one by one in this way.

Chemical Analyses

The signaling compounds were identified by using the Finnigan SSQ 7000 GC-MS system, including a Varian 3400 GC. One DB-5 and one DB-Wax silica capillary column (30 m $\times$ 0.25 mm ID, 0.25-μm film thickness) were used with a temperature program of 70°C (1 min), increased by 10°C/min to 140°C, then by 1°C/min up to 150°C, by 2°C/min up to 170°C, by 5°C/min up to 200°C, and thereafter maintained constant at 200°C for 17 min. The split/splitless injector temperature was 225°C, and the splitless period was 30 sec. Helium was used as the carrier gas with an inlet pressure of 5 psi. Electron ionization mass spectra were determined at 70 eV with the ion source at 150°C. When the sample had been injected, the desorption of the sex pheromone components and of the internal standards from the SPME fiber was checked by subsequent injection into a split/splitless injector of a second GC for 30 sec of splitless time at an injector temperature of 225°C. Total desorption of the compounds occurred during the first injection.

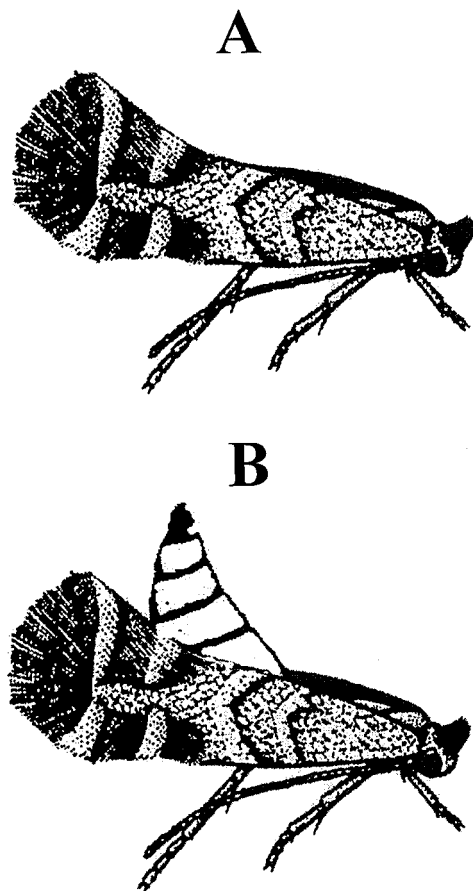


FIG. 1. Postures of virgin *Phyllonorycter emberizaepenella* females: (A) resting, (B) calling.

The volatiles collected from females during their calling and noncalling periods were compared chromatographically, and only those coming exclusively from calling periods were analyzed. The compounds selected for analysis were identified by comparison of their mass spectral data and GC-retention times with the corresponding data of synthetic standards.

Chemicals

All isomers of 10,12-tetradecadienols and their acetates used as GC-MS standards were available from previous research, whereas the 8,10-tetradecadienols and

their acetates were synthesized at the Department of Chemistry, Organic Chemistry, The Royal Institute of Technology, Stockholm. The synthetic intermediates as well as the products were characterized with a Bruker AM 400 spectrometer (NMR spectra were recorded in $CDCl_3$), and the GC-MS system is described in the section above (Chemical Analyses).

8-Bromooctanol (**2**; Figure 2) was prepared according to Rossi *et al.* (1981), Gardette *et al.* (1983), and Tellier (1990). 8-Hydroxyoctyl-triphenylphosphonium bromide (**3**; Figure 2) was synthesized as in Vinczer *et al.* (1987). (8*E*,10*E*)-8,10-tetradecadienol (**4**; Figure 2) and (8*Z*, 10*E*)-8,10-tetradecadienol (**5**; Figure 2) were made according to Lo and Shiao, (1986). The products were obtained in a mixture of **4** (40%) and **5** (60%). *ZZ* and *EZ* isomers were not detected by GC-MS. Isomers **4** and **5** were separated by chromatography on silica gel impregnated with 15% $AgNO_3$. Finally, 99.0% isomerically pure **4** was derived from a urea inclusion complex, and **5** was obtained in 99.2% purity by crystallization from pentane at -30°C.

*(8*E*,10*E*)-8,10-Tetradecadienol **4***. ^{1}H NMR: δ: 5.97 (m, 2H, $-$CH=C$\underline{\text{H}}$-C$\underline{\text{H}}$=CH$-$), 5.54 (m, 2H, $-$C$\underline{\text{H}}$=CH-CH=C$\underline{\text{H}}-$), 3.61 (t, $J = 6.6$ Hz, 2H, $-$C$\underline{\text{H}}_2$-OH), 2.02 (m, 4H, $-$C$\underline{\text{H}}_2$-CH=), 1.24–1.54 (m, 12H), 0.89 (t, $J = 5.7$ Hz, 3H, $-$CH$_2$-C$\underline{\text{H}}_3$). EI-MS [m/z (relative intensity)]: 210 (M^+, 34), 192 (6), 135 (11), 121 (18), 111 (7), 110 (14), 109 (17), 108 (7), 107 (12), 98 (10), 97 (10), 96 (45), 95 (32), 94 (13), 93 (24), 91 (8), 83 (9), 82 (41), 81 (77), 80 (22), 79 (42), 77 (8), 69 (9), 68 (26), 67 (100), 55 (32), 54 (34), 43 (12), 41 (30).

*(8*Z*,10*E*)-8,10-Tetradecadienol **5***. ^{1}H NMR: δ: 6.28 (dd, $J = 11.0$, 14.8 Hz, 1H, $-$C^{8}H=C^{9}H-C$^{10}\underline{\text{H}}$=C^{11}H$-$), 5.93 (dd, $J = 10.9$, 11.0 Hz, 1H, $-$C^{8}H=C$^9\underline{\text{H}}$-C^{10}H=C^{11}H$-$), 5.63 (dt, $J = 7.3$, 15.0 Hz, 1H, $-$C^{8}H=C^{9}H-C^{10}H=C$^{11}\underline{\text{H}}-$), 5.31 (dt, $J = 7.4$, 10.8 Hz, 1H, $-$C$^8\underline{\text{H}}$=C^{9}H-C^{10}H=C^{11}H$-$), 3.61 (t, $J = 6.5$ Hz, 2H, $-$C$\underline{\text{H}}_2$-OH), 2.06-2.16 (m, 4H, $-$C$\underline{\text{H}}_2$-CH=), 1.28-1.65 (m, 12H), 0.89 (t, $J = 7.2$ Hz, 3H, $-$CH$_2$-C$\underline{\text{H}}_3$). EI-MS [m/z (relative intensity)]: 210 (M^+, 34), 192 (6), 135 (8), 122 (7), 121 (18), 111 (7), 110 (15), 109 (17), 108 (7), 107 (13), 98 (10), 97 (10), 96 (43), 95 (33), 94 (13), 93 (25), 91 (7), 83 (9), 82 (38), 81 (75), 80 (21), 79 (38), 77 (8), 69 (10), 68 (25), 67 (100), 55 (31), 54 (33), 43 (12), 41 (28).

*(8*E*,10*E*)-8,10-Tetradecadienyl Acetate (**6**; Figure 2).* Pyridine (2 ml) and acetic anhydride (0.5 ml) were added to compound **4** (0.200 g, 0.95 mmol) at room temperature, and the mixture was left in a refrigerator overnight. The next day it was poured into ice-water and extracted with hexane. The extract was dried and chromatographed to yield **6** (0.205 g, 91%). No loss in isomeric purity was observed. ^{1}H NMR: δ: 5.97 (m, 2H, $-$CH=C$\underline{\text{H}}$-C$\underline{\text{H}}$=CH$-$), 5.54 (m, 2H, $-$C$\underline{\text{H}}$=CH-CH=C$\underline{\text{H}}-$), 4.01 (t, $J = 6.5$ Hz, 2H, $-$C$\underline{\text{H}}_2$-COO$-$), 2.08 (m, 4H, $-$C$\underline{\text{H}}_2$-CH=), 2.04 (s, 3H, C$\underline{\text{H}}_3$-COO$-$), 1.22-1.50 (m, 12H), 0.88 (t, $J = 5.6$ Hz, 3H, $-$CH$_2$-C$\underline{\text{H}}_3$). EI-MS [m/z (relative intensity)]: 252 (M^+, 44), 192 (33), 163 (7), 149 (14), 136 (9), 135 (27), 124 (7), 123 (7), 122 (14), 121 (40), 110 (28), 109 (21), 108 (16),

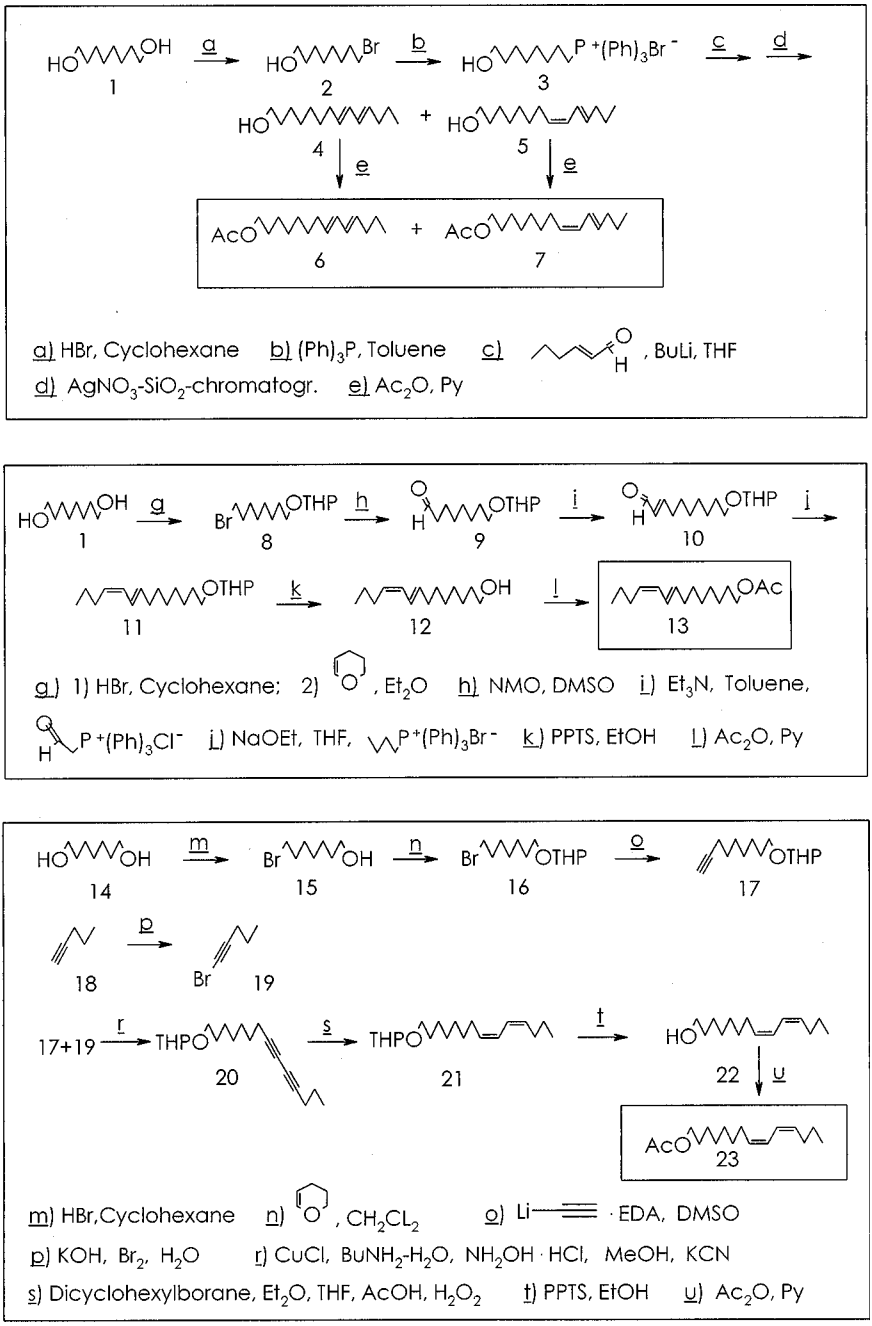


Fig. 2. Schemes of the syntheses of 8,10-tetradecadienols and their acetates.

107 (22), 97 (8), 96 (44), 95 (33), 94 (23), 93 (38), 91 (8), 83 (7), 82 (38), 81 (72), 80 (35), 79 (7), 78 (8), 69 (10), 68 (22), 67 (100), 66 (8), 55 (28), 54 (24), 43 (51), 41 (24).

*(8Z,10*E*)-8,10-Tetradecadienyl Acetate (**7**; Figure 2).* Acetylation of **5,** as described above for **4**, gave **7** in 88% yield. Minor isomerization products were separated by liquid chromatography on $AgNO_3$/silica gel to yield 99% isomerically pure acetate **7**. ^{1}H NMR: δ: 6.28 (dd, $J = 11.0, 14.8$ Hz, 1H, $-C^8H{=}C^9H$-$C^{10}\underline{H}{=}C^{11}H-$), 5.93 (dd, $J = 10.9, 11.0$ Hz, 1H, $-C^8H{=}C^9\underline{H}$-$C^{10}H{=}C^{11}H-$), 5.65 (dt, $J = 7.3, 15.0$ Hz, 1H, $-C^8H{=}C^9H$-$C^{10}H{=}C^{11}\underline{H}-$), 5.30 (dt, $J = 7.4, 10.8$ Hz, 1H, $-C^8\underline{H}{=}C^9H$-$C^{10}H{=}C^{11}-$), 4.02 (t, $J = 6.6$ Hz, 2H, $-C\underline{H}_2$-COO–), 2.10 (m, 4H, $-C\underline{H}_2$-CH=), 2.03 (s, 3H, $C\underline{H}_3$-COO–), 1.26–1.40 (m, 12H), 0.90 (t, $J = 6.8$ Hz, 3H, $-CH_2$-$C\underline{H}_3$). EI-MS [m/z (relative intensity)]: 252 (M^+, 37), 192 (33), 163 (7), 149 (18), 136 (9), 135 (25), 124 (7), 122 (15), 121 (42), 110 (29), 109 (19), 108 (15), 107 (22), 97 (8), 96 (43), 95 (35), 94 (25), 93 (38), 91 (7), 83 (8), 82 (42), 81 (77), 80 (35), 79 (63), 78 (7), 77 (8), 69 (9), 68 (23), 67 (100), 66 (7), 55 (31), 54 (26), 43 (53), 41 (28).

8-Bromo-1-(2-tetrahydropyranyloxy)-octane (**8**; Figure 2) was synthesized as described by Miyashita et al. (1977). 8-(2-Tetrahydropyranyloxy)-octanal (**9**; Figure 2) was made based on Ando et al. (1988). 4-(*E*)-10-(2-Tetrahydropyranyloxy)-2-decenal(**10**, Figure 2) was made according to Ando et al. (1985). (8*E*,10*Z*)-1-(2-Tetrahydropyranyloxy)-8,10-tetradecadiene (**11**, Figure 2) was synthesized based on the methods published by Vinczer et al. (1988).

*(8*E*,10*Z*)-8,10-Tetradecadienol (**12**; Figure 2).* Deprotection of **11** (2.2 g, 7.5 mmol) was performed in EtOH (25 ml) at 55°C for 6 hr in the presence of pyridinium tosylate (188 mg, 0.75 mmol) as a catalyst. Evaporation of the ethanol and chromatography yielded a crude product from which 0.86 g (55%) 97% isomerically pure **12** was obtained after crystallization from pentane. Chromatography on $AgNO_3$-impregnated silica gel enriched the isomeric purity up to 99.5%. ^{1}H NMR: δ: 6.32 (dd, $J = 10.9, 15.0$ Hz, 1H, $-C^8H{=}C^9\underline{H}$-$C^{10}H{=}C^{11}H-$), 5.95 (dd, $J = 10.6, 10.9$ Hz, 1H, $-C^8H{=}C^9H$-$C^{10}\underline{H}{=}C^{11}H-$), 5.64 (dt, $J = 7.0, 15.1$ Hz, 1H, $-C^8\underline{H}{=}C^9H$-$C^{10}H{=}C^{11}H-$), 5.30 (dt, $J = 7.5, 10.9$ Hz, 1H, $-C^8H{=}C^9H$-$C^{10}H{=}C^{11}\underline{H}-$), 3.63 (t, $J = 6.9$ Hz, 2H, $-C\underline{H}_2$-OH), 1.90–2.22 (m, 4H, $-C\underline{H}_2$-CH=), 1.21–1.72 (m, 12H), 0.93 (t, $J = 7.6$ Hz, 3H, $-CH_2$-$C\underline{H}_3$). EI-MS [m/z (relative intensity)]: 210 (M^+, 49), 192 (13), 176 (13), 167 (8), 135 (9), 125 (8), 123 (17), 122 (7), 121 (25), 110 (17), 109 (23), 107 (15), 98 (9), 97 (18), 96 (47), 95 (35), 94 (24), 93 (19), 91 (17), 87 (10), 85 (12), 83 (8), 82 (44), 81 (82), 80 (24), 79 (64), 71 (23), 70 (7), 69 (15), 68 (24), 67 (100), 66 (9), 55 (25), 54 (32), 41 (17).

*(8*E*,10*Z*)-8,10-Tetradecadienyl acetate (**13**; Figure 2).* The alcohol **12** was acetylated in the manner described above for compounds **6** and **7**. Acetylation gave **13** in 87% yield. Minor isomerization products were separated by chromatography on $AgNO_3$-impregnated silica gel to yield 99.5% isomerically pure acetate

13. ^{1}H NMR: δ: 6.32 (dd, $J = 10.9, 15.0$ Hz, 1H, $-C^8H{=}C^9\underline{H}\text{-}C^{10}H{=}C^{11}H-$), 5.95 (dd, $J = 10.6, 10.9$ Hz, 1H, $-C^8H{=}C^9H\text{-}C^{10}\underline{H}{=}C^{11}H-$), 5.64 (dt, $J = 7.0, 15.1$ Hz, 1H, $-C^8\underline{H}{=}C^9H\text{-}C^{10}H{=}C^{11}H-$), 5.30 (dt, $J = 7.5, 10.9$ Hz, 1H, $-C^8H{=}C^9H\text{-}C^{10}H{=}C^{11}\underline{H}-$), 4.05 (t, $J = 6.8$ Hz, 2H, $-C\underline{H}_2\text{-}COO-$), 1.90–2.16 (m, 4H, $-C\underline{H}_2\text{-}CH{=}$), 2.04 (s, 3H, $C\underline{H}_3\text{-}COO-$), 1.30–1.70 (m, 12H), 0.91 (t, $J = 7.3$ Hz, 3H, $-CH_2\text{-}C\underline{H}_3$). EI-MS [$m/z$ (relative intensity)]: 252 (M^+, 39), 192 (32), 149 (12), 136 (8), 135 (26), 123 (7), 122 (14), 121 (40), 110 (28), 109 (19), 108 (15), 107 (21), 97 (7), 96 (42), 95 (32), 94 (20), 93 (36), 91 (8), 83 (7), 82 (36), 81 (72), 80 (34), 79 (58), 69 (8), 68 (21), 67 (100), 55 (30), 54 (24), 43 (52), 41 (27).

7-Bromoheptanol (**15**; Figure 2) was obtained by a procedure similar to the one described above for 8-bromooctanol, **2**. For 7-Bromo-1-(2-tetrahydropyranyloxy)-heptane (**16**; Figure 2), the alcohol **15** was protected as its THP–ether by the usual method, which gave **16** in 95% yield. ^{1}H NMR: δ: 4.57 (t, $J = 3$ Hz, 1H), 3.31–3.86 (m, 4H, $-C\underline{H}_2\text{-}O-$), 3.63 (t, $J = 6.6$ Hz, 2H, $-C\underline{H}_2Br$), 1.30–1.81 (m, 16H). 1-(2-Tetrahydropyranyloxy)-8-nonyne (**17**; Figure 2) was prepared according to Rossi et al. (1981); Gardette et al. (1983).

*1-Bromo-1-pentyne (**19**; Figure 2).* Potassium hydroxide (67.2 g, 1.2 mol) was dissolved in water (370 ml). Bromine (32 g, 0.2 mol) was added dropwise at 0°C during 30 min, and 1-pentyne (**18**) (13.0 g, 0.19 mol) was then added to the solution, while the temperature was maintained at 0°C. The reaction mixture was stirred for 14 hr at room temperature. The product was extracted with a pentane–diethyl ether mixture (1:1), and the extract was dried and concentrated. Distillation gave 23.9 g of **19,** an 85% yield. ^{1}H NMR: δ: 2.18 (t, $J = 7.0$ Hz, 2H, $-C\underline{H}_2\text{-}C{\equiv}$), 1.54 (td, $J = 7.3, 14.3$ Hz, 2H, $-C\underline{H}_2-$), 0.98 (t, $J = 7.4$ Hz, 3H, $-C\underline{H}_3$).

1-(2-Tetrahydropyranyloxy)-8,10-tetradecadiyne (**20**; Figure 2) was obtained by a procedure described by Svirskaya et al. (1980); Chisholm et al. (1985); De Jarlais and Emken (1987). (8*Z*,10*Z*)-1-(2-Tetrahydropyranyloxy)-8,10-tetradecadiene (**21**; Figure 2) was synthesized according to Svirskaya et al. (1980); Chisholm et al. (1985); De Jarlais and Emken, (1987).

*(8Z,10Z)-8,10-Tetradecadienol (**22**; Figure 2).* Compound **21** (1.78 g, 6.1 mmol) was added to pyridinium tosylate in ethanol (25 ml). The mixture was warmed at 55°C for 4 hr. Evaporation of the ethanol, followed by chromatography, yielded 1.2 g (80%) of **22** with an isomeric purity of 97%. After crystallization from pentane at −20°C, no isomers were detected by GC. ^{1}H NMR: δ: 6.28 (m, 2H, $-CH{=}C\underline{H}\text{-}C\underline{H}{=}CH-$), 5.47 (m, 2H, $-C\underline{H}{=}CH\text{-}CH{=}C\underline{H}-$), 3.65 (t, $J = 6.7$ Hz, 2H, $-C\underline{H}_2\text{-}O-$), 2.18 (m, 4H, $-C\underline{H}_2\text{-}CH{=}$), 1.56 (m, 2H), 1.26–1.46 (m, 10H), 0.94 (t, $J = 7.3$ Hz, 3H, $-C\underline{H}_3$). EI-MS [m/z (relative intensity)]: 210 (M^+, 26), 192 (8), 135 (12), 122 (7), 121 (19), 111 (8), 110 (18), 109 (15), 108 (8), 107 (12), 98 (12), 97 (11), 96 (45), 95 (34), 94 (14), 93 (25), 91 (7), 83 (9), 82 (42), 81 (84), 80 (22), 79 (42), 77 (8), 69 (10), 68 (27), 67 (100), 55 (33), 54 (35), 43 (11), 41 (31).

*(8Z,10Z)-8,10-Tetradecadienyl acetate (**23**; Figure 2).* The acetylation procedure described above gave the acetate **23** in 92% yield. No isomerization occurred during the acetylation. ^{1}H NMR: δ: 6.28 (m, 2H, —CH=C<u>H</u>-C<u>H</u>=CH—), 5.56 (m, 2H, —C<u>H</u>=CH-CH=C<u>H</u>—), 4.08 (t, $J = 6.7$ Hz, 2H, —C<u>H$_2$</u>-O—), 2.17 (m, 4H, —C<u>H$_2$</u>-CH=), 2.08 (s, 3H, C<u>H$_3$</u>COO—), 1.34–1.67 (m, 12H), 0.94 (t, $J = 7.4$ Hz, 3H, —C<u>H$_3$</u>). EI-MS [m/z (relative intensity)]: 252 (M^+, 24), 193 (8), 192 (43), 163 (7), 149 (13), 136 (10), 135 (26), 124 (6), 123 (7), 122 (14), 121 (39), 110 (27), 109 (18), 108 (16), 107 (22), 97 (8), 96 (40), 95 (35), 94 (24), 93 (40), 91 (9), 83 (8), 82 (40), 81 (81), 80 (35), 79 (66), 78 (7), 77 (8), 69 (11), 68 (23), 67 (100), 66 (8), 55 (32), 54 (37), 43 (54), 41 (28).

Trapping Tests

The trapping tests using virgin calling females were carried out in the vicinity of Vilnius in eastern Lithuania from April 20 to May 20, 1990. Two-day-old virgin calling females were placed in a 30-ml wire mesh cylinder containing a sponge with a solution of 5% of sucrose in water that was replaced every two days. The container was placed in an opaque white Atracon A delta trap containing an exchangeable bottom (11 × 18 cm), coated with Pestifix glue. Ten traps, each containing one female, were placed about 1 m above the ground on a branch of common honeysuckle shrub with a distance of >10 m between traps and inspected daily. Females were replaced by young females when they reached age 10 days of or were found to be not calling.

The field tests of potential synthetic sex pheromone components were carried out at the same locality in the vicinity of Vilnius from May 19 to June 15, 2000. The compounds were dissolved in hexane (Merck p.a.) and, either alone or in binary mixtures, soaked from the inside into the walls of red rubber tube dispensers (18 × 15 mm). Samples of 200 μg 8*E*,10*E*–14:OAc/dispenser; 0.2 μg 8*E*,10*E*–14:OH/dispenser; 200 μg 8*E*,10*E*–14:OAc + 0.2 μg 8*E*,10*E*–14:OH/dispenser and 50 μl hexane/dispenser (as a control) were tested. Five replications of each lure were used. In order to diminish the impact of biotopic variation on the trapping results, all traps of one replication set were moved to the location of traps of another replication set every five days.

RESULTS

Biological Characteristics of Ph. emberizaepenella Sample. After the winter diapause, 172 moths were obtained from pupae collected in the autumn of 1989 (42% of the pupae were infected by parasites and 12% died due to other causes). In all, 748 moths emerged from the mined leaves collected in the autumns of 1996–1998, and 82 moths emerged from the pupae collected in the summer of 1999. All of the adult moths were females. In addition to the individuals obtained from

pupae, we collected 134 moths outdoors from April 20 through May 20, 1990 with an entomological net, and even in this group all specimens were females. Under laboratory conditions the average length of life of the adult female was 15 ± 5 days ($N = 37$).

Parthenogenetic Mode of Reproduction. Normally developed caterpillars emerged from unfertilized eggs laid by virgin females of *Ph. emberizaepenella*. Host plant twigs with leaves containing offspring of three *Ph. emberizaepenella* females remained suitable as food sources for the larvae long enough to allow them to form typical pupae. Seventeen adults of the F_1 generation were obtained from the pupae, and all of them were females.

Calling Behavior of Females. By observing the behavior of virgin females in nature, we established that they preferred to stay on the underside of the host plant leaves, where they remained stationary, while exhibiting the calling posture. The most typical elements of this posture were the following: wings slightly spread and lowered, abdominal tip dorsally curved and distal abdominal segments protruded, and antennae placed along the wings (Figure 1B).

Under laboratory conditions, females started to call on the second day after emergence. The peak of activity was registered 0.5–3 hr after the beginning of the light phase. Nearly 100% of the females ($N = 37$) were active during the calling peak. The total duration of the active period within the group of 2- to 4-day-old females was about 6 hr/day (Figure 3).

Chemical Identification of Compounds Collected from Calling Females. Two compounds were found to be specific for the calling periods of virgin

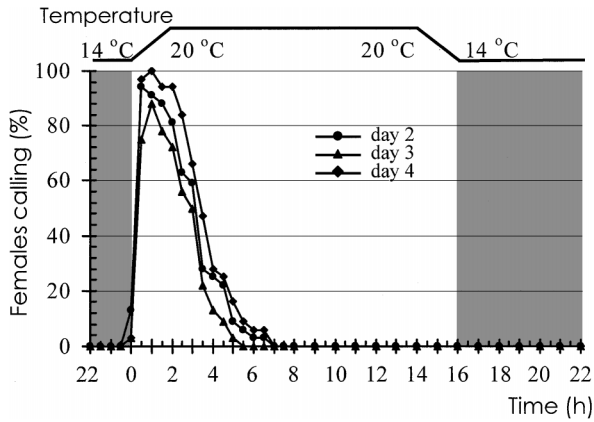


FIG. 3. Diurnal rhythm of calling activity of virgin *Phyllonorycter emberizaepenella* females under laboratory conditions. Shaded areas: scotophase, white area: photophase. The females were not found calling during the first day after emergence. $N = 32$ females in each day of the observation.

Ph. emberizaepenella females, when GC-MS chromatograms from calling and noncalling periods were compared (Figure 4AB). One of these compounds showed m/z 61 but not m/z 60, typical diagnostic ions of acetates and fatty acids, respectively. The diagnostic ion, m/z 192 (M-60), indicated di-unsaturation and a chain length of C_{14}. In addition, the well-expressed molecular ion m/z 252 ($M^{+\cdot}$) confirmed that the compound was a tetradecadienyl acetate and indicated that the double bonds were conjugated (Figure 5A).

The second compound showed a fragmentation pattern similar to that of the tetradecadienyl acetate. The presence of the diagnostic ions m/z 31 and m/z 210 ($M^{+\cdot}$) instead of m/z 61 and m/z 252 ($M^{+\cdot}$), respectively, indicated that the second compound was a tetradecadienol (Figure 5B).

In all unsaturated sex pheromone components identified from species of the genus *Phyllonorycter*, double bonds occurred only at even positions (Mozūraitis et al., 1999; Arn et al., 2001). Therefore, we compared the retention times of the two compounds collected from the calling females with those of synthetic 8,10- and 10,12-tetradecadienols and their acetates. This comparison showed that the volatiles released by the *Ph. emberizaepenella* females were 8*E*,10*E*–14:OAc and 8*E*,10*E*–14:OH (Table 1). A comparison of the mass spectra of the compounds collected from calling females with those of synthetic standards confirmed the results of the GC analyses.

Volatiles from all 12 calling females contained a trace amount of 8*E*,10*E*–14:OH relative to the acetate, detected only by using the most abundant diagnostic ions m/z 67, m/z 81 and m/z 210 ($M^{+\cdot}$) (Figure 5B).

Trapping Experiments. During the trapping period, which was about twice as long as the average lifetime of adult females, neither traps containing virgin calling females nor lures made up with potential sex pheromones captured any males, in spite of the large number of moths of this species in the habitat. These facts indicated that the probability of finding a male among moths of the first generation of this population was extremely small, close to zero. Consequently, 8*E*,10*E*–14:OAc and the corresponding alcohol have to be considered as potential sex pheromone components of this leaf-miner species.

DISCUSSION

The development of adults from unfertilized eggs, as well as the absence of males in both generations, confirmed the previous report (Būda et al., 1991) on parthenogenesis of *Ph. emberizaepenella* species. When only females develop from unfertilized eggs, the term thelytoky is applicable for characterizing the parthenogenesis. Regular thelytoky is rare among the Lepidoptera, but it has been found in some species belonging to the families Psychide, Tineidae, and Lymantridae (reviews: Cockayne, 1938; Suomalainen, 1950; Went, 1984). As far

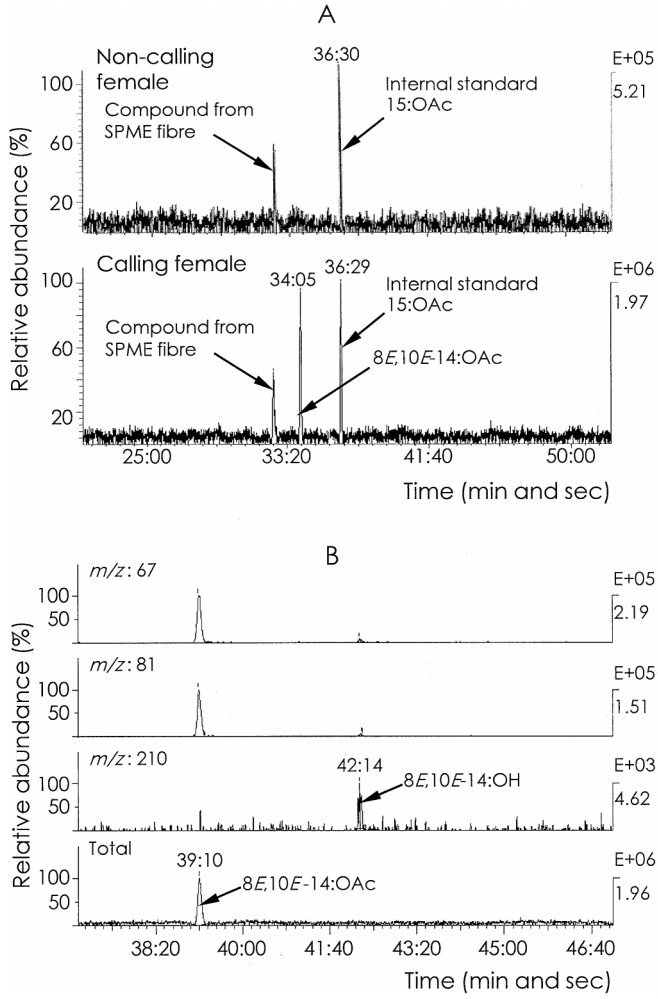


FIG. 4. Total and selected ion chromatogram records of volatiles collected from one *Phyllonorycter emberizaepenella* female. (A) Total ion chromatogram in the range m/z 30–400; Noncalling and calling indicate that volatiles were obtained during noncalling and calling periods of a female, respectively; DB-5 fused silica capillary column (30 m $\times$ 0.25-mm ID, 0.25-μm film thickness). (B) Selected ion chromatograms where m/z 67 and m/z 81 ion fragments indicate unsaturation; m/z 210 is the molecular ion characteristic of tetradecadienols; Total represents chromatogram in the ion range m/z 30–400; HP-Wax fused silica capillary column (30 m $\times$ 0.25 mm ID, 0.25-μm film thickness); m/z indicates the ratio of the mass of the ion to the number of charges on it. The numbers at the upper-right corner of the chromatograms, for example, E+05 5.21, represent the number of counts related to the abundance of the ions formed and E+05 5.21 has to be read as 5.21×10^5.

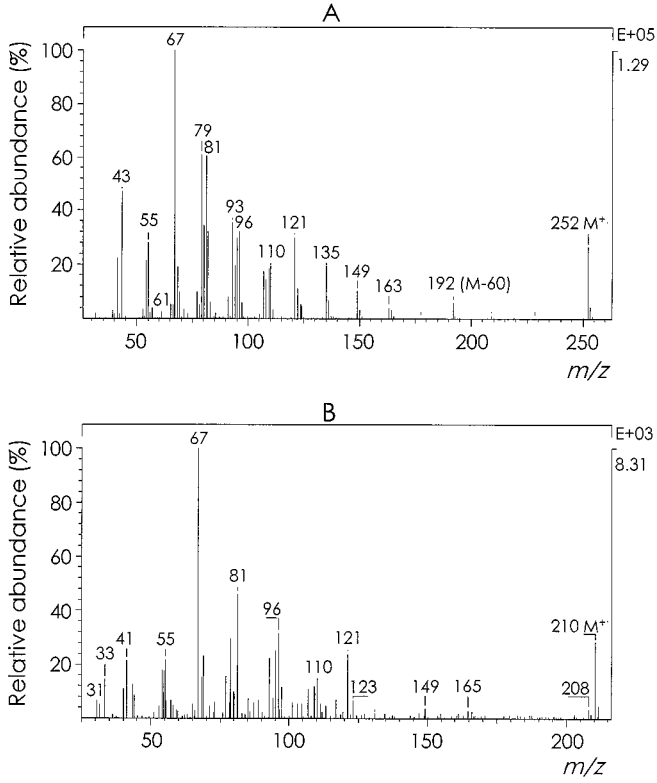


FIG. 5. Mass spectra of the compounds obtained only during calling periods of *Phyllonorycter emberizaepenella* females: (A) 8*E*,10*E*-14:OAc; (B) 8*E*,10*E*-14:OH; *m*/*z* indicates the ratio of the mass of the ion to the number of charges on it; $M^{+\cdot}$ represents the molecular ion; the number at the upper-right corner of the mass spectrum, e.g., E+0.5 1.29, represents the number of counts related to the abundance of the ions formed and E+0.5 1.29 has to be read as 1.29×10^5.

as we know, *Ph. emberizaepenella* is the first case of thelytokous parthenogenesis found in the Gracillariidae family.

Ph. emberizaepenella females demonstrated a calling posture similar to that of other species of the same genus that we have investigated: *Ph. ulmifoliella* (Hb.) (Mozūraitis et al., 1997), *Ph. acerifoliella* (Z.) and *Ph. heegerella* (Z.) (Mozūraitis et al., 2000), and *Ph. sorbi* (Frey.) and *Ph. strigulatella* (Lien *et* Z.) (Mozūraitis, 2000). This posture is also similar to that of the pyralids, *Ephestia* (=*Anagasta*) *kuehniella* (Z.) (Traynier, 1968) and *Plodia interpunctella* (Hb.) (Brady and Smithwich, 1968).

TABLE 1. RETENTION TIMES OF COMPOUNDS OBTAINED FROM CALLING *Phyllonorycter enberizaepenella* FEMALES, AND SYNTHETIC 8,10- AND 10,12-TETRADECADIENOLS AND THEIR ACETATES, WHEN PASSING THROUGH TWO CAPILLARY COLUMNS

Compound	Retention time (min:sec)	
	Polar column (HP-wax)	Unpolar column (DB-5)
From female		
(8*E*,10*E*)-8,10-tetradecadienyl acetate	39:10	34:05
(8*E*,10*E*)-8,10-tetradecadienol	42:14	26:03
Synthetic		
(8*Z*,10*Z*)-8,10-tetradecadienyl acetate	38:44	33:46
(8*Z*,10*E*)-8,10-tetradecadienyl acetate	37:24	32:38
(8*E*,10*Z*)-8,10-tetradecadienyl acetate	38:08	33:10
(8*E*,10*E*)-8,10-tetradecadienyl acetate	39:10	34:05
(8*Z*,10*Z*)-8,10-tetradecadienol	41:48	25:35
(8*Z*,10*E*)-8,10-tetradecadienol	40:51	23:49
(8*E*,10*Z*)-8,10-tetradecadienol	41:19	24:30
(8*E*,10*E*)-8,10-tetradecadienol	42:14	26:02
(10*Z*,12*Z*)-10,12-tetradecadienyl acetate	40:18	34:39
(10*Z*,12*E*)-10,12-tetradecadienyl acetate	39:23	33:46
(10*E*,12*Z*)-10,12-tetradecadienyl acetate	40:05	34:31
(10*E*,12*E*)-10,12-tetradecadienyl acetate	39:47	34:18
(10*Z*,12*Z*)-10,12-tetradecadienol	42:53	26:43
(10*Z*,12*E*)-10,12-tetradecadienol	42:11	25:35
(10*E*,12*Z*)-10,12-tetradecadienol	42:43	26:31
(10*E*,12*E*)-10,12-tetradecadienol	42:30	26:15

A distinct diurnal rhythm of the calling behavior was established on the basis of the calling posture. *Ph. emberizaepenella* females were active after the start of the light period of a day, which is a common calling time for other species of the same genus [*Ph. ulmifoliella* (Mozūraitis et al., 1997); *Ph. ringoniella* (Matsumura) (Boo and Jung, 1998); *Ph. acerifoliella* and *Ph. heegerella*, (Mozūraitis et al., 2000), *Ph. sorbi* and *Ph. strigulatella* (Mozūraitis, 2000)] and for some species of other families (Dreisig, 1986; McNeil, 1991, Tóth et al., 1995).

Both compounds identified from calling *Ph. emberizaepenella* females were new for the family Gracillariidae, but their chemical structures corresponded to the general pattern of the structures determined for sex pheromones of other *Phyllonorycter* species. These compounds were aliphatic C_{12}–C_{14} straight-chain, saturated, mono- or di-unsaturated acetates or alcohols, with *Z* or *E* double bonds occurring in positions 4, 8, 10, and 12 (Mozūraitis et al., 1999; Arn et al., 2001).

Assuming that both compounds were collected from virgin females only during calling periods, and their chemical structures corresponded to the general pattern of the ones determined for sex pheromones from other *Phyllonorycter* species, it is possible that 8*E*,10*E*–14:OAc alone or in a binary mixture with a

corresponding alcohol is a sex pheromone conponent of *Ph. emberizaepenella* species.

All these characteristics of chemical communication of *Ph. emberizaepenella* females are very similar or even identical to those of Lepidoptera with the usual amphimictic mode of reproduction. In spite of the parthenogenetic reproduction during which complete reduction of sexual behavior might be expected, the females of this leafmining species exhibited the calling posture and released potential sex pheromone with the diurnal rhythm of such behavior that is inherent in the process. Why do parthenogenetically reproducing females still call and synthesize and release potential sex pheromone in the absence of males?

A reduction of sexual behavior involves changes in the physiology of an organism. From the viewpoint of the theory of optimality (Rozen, 1969), these changes are justified only when they are more beneficial than the cost of carrying out all necessary modifications. The benefit of the absence of male finding would include both resources and time saved from biosynthesis of a sex pheromone, sex pheromone release, and exposure to potential natural enemies such as egg parasitoids that use moth sex pheromones to locate resources. While the costs include those for turning off sex pheromone biosynthesis and calling behavior.

The greatest benefit would be achieved by turning off both calling behavior and biosynthesis of sex pheromones. Unfortunately, there are no data available on these processes in the family Gracillariidae. Thus, our discussion must be based on knowledge of other lepidopteran taxa.

In most investigated lepidopteran species, sex pheromone biosynthesis requires pheromone biosynthesis activating neuropeptide (PBAN) or PBAN-like compounds (Ramaswamy, 1997; Tillman et al., 1999). To turn off production of a single peptide would not be complicated or costly if its role is not multifunctional. However, in some species, pheromone glands are able to produce sex pheromone through the diel period without the presence of PBAN, and this competence is achieved as a result of developmental–metamorphic events (Tang et al., 1989, 1991). The prediction of a mechanism and the cost of switching off sex pheromone biosynthesis in these species is unclear.

Calling in Lepidoptera is usually controlled by neural outputs from the brain or other neuronal centers (Sasaki et al., 1983; Itagaki and Corner, 1986; Tang et al., 1987; Christensen et al., 1994). Thus, the stopping of calling behavior should not be costly. However, more complex regulation of pheromone release occurs in two migratory noctuid moth species. In the true armyworm moth, *Pseudatelia unipunctata* (Haworth), juvenile hormone (JH) must be continuously present to maintain calling behavior once initiated (Cusson and McNeil, 1989a), and the same hormone is important for ovarian development (Cusson and McNeil, 1989b). The similar function of JH has been demonstrated in black cutworm moth, *Agrotis ipsilon* (Hufnagel) (Gadenne et al., 1993; Picombon et al., 1995). Turning off calling by inhibiting production of JH would lead to termination of ovarian development, thus

disturbing reproduction. In such an event, the cost for termination of calling and sex pheromone production obviously would overcome the benefit, and it is most likely that females would continue to release signaling compounds despite the complete lack of males. Possibly, similar regulation of pheromone release might occur in parthenogenetically reproducing *Ph. emberizaepenella* species.

The theoretical speculation that in thelytoky, individuals obtain advantages due to a reduction in their sexual behavior when there is no need to find a sexual partner clearly needs re-examination.

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HOST-RECOGNIZING KAIROMONES FOR PARASITIC WASP, *Anisopteromalus calandrae*, FROM LARVAE OF AZUKI BEAN WEEVIL, *Callosobruchus chinensis*

JUNKO ONODERA,[1,3] SHIGERU MATSUYAMA,[1] TAKAHISA SUZUKI,[1,*] and KOICHI FUJII[2]

[1]*Institute of Applied Biochemistry*
[2]*Institute of Biological Sciences*
University of Tsukuba
Tsukuba, Ibaraki 305-8572, Japan

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Abstract—Host-recognizing kairomones for the stinging behavior of the parasitic wasp, *Anisopteromalus calandrae*, were identified on host azuki bean weevil larvae, *Callosobruchus chinensis* (L.). The kairomones were extracted with acetone from Chinese green beans, from which emerged wasps and host weevils had been removed. The kairomones are a mixture of triacylglycerols and fatty acids, each of which is separately active, and with no observable synergistic effect between them. These compounds are known to be constituents of an oviposition-marking pheromone of host azuki bean weevils. However, they differ from the previously reported saturated hydrocarbons and diacylglycerols of the kairomone that another parasitic wasp, *Dinarmus basalis*, uses for the host recognition of *C. chinensis*. Thus, *A. calandrae* and *D. basalis* selectively utilize different constituents of the oviposition-marking pheromone of *C. chinensis* as host-recognizing kairomones.

Key Words—*Anisopteromalus calandrae*, parasitic wasp, oviposition-marking pheromone, host-recognizing kairomone, azuki bean weevil, *Callosobruchus chinensis*.

INTRODUCTION

The azuki bean weevil, *Callosobruchus chinensis* (L.) (Coleoptera: Bruchidae) is a serious pest of stored *Vigna* legumes, such as azuki beans (*V. angularis*), cowpea beans (*V. catiang*), and Chinese green beans (*V. radiata*). The physiology

* To whom correspondence should be addressed. E-mail: tksuzuki@agbi.tsukuba.ac.jp
[3] Present address: Advantec Toyo Kaisha Ltd., Kotobuki 1-5-10, Taito-ku Tokyo 111-0042, Japan.

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and ecology of this multivoltine insect has been widely researched, and it had also been used as a test insect against insecticides. Consequently, the chemical ecology of this weevil has been well studied, including that of oviposition-marking pheromones (Ohshima et al., 1973; Sakai et al., 1986; Honda and Ohsawa, 1990), a copulation-release pheromone (Tanaka et al., 1981), and oviposition stimulants (Ueno et al., 1990; Matsumoto et al., 1994; Tebayashi et al., 1995).

The parasitic wasp, *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae), is an important natural parasite of larvae of the maize weevils, *Sitophilus zeamais* Motschulsky, a major pest of stored corn, *Zea mays* L. (Williams and Floyd, 1971; Arbogast and Mullen, 1990; Smith, 1992). This wasp also attacks several other bruchid weevils, including azuki bean weevils (Utida and Nagasawa, 1949; Okamoto, 1971, 1972), rice weevils, *S. oryzae* (L.) (Press et al., 1984; Cline et al., 1985), granary weevils, *S. granarius* (L.) (Ghani and Sweetman, 1955; van den Assem et al., 1984), and Mexican bean weevils, *Zabrotes subfasciatus* Bohman.

Adults of *A. calandrae* are sexually dimorphic; females are entirely black and ca. 2.5 mm in length while males have partly milk-white abdomens and are ca. 2.0 mm in length. When an azuki bean or Chinese green bean containing host larvae is presented to a female, the following sequential behaviors are observed: the female walks and examines the surface of the bean with antennal tapping, bends her abdomen vertically, stings her ovipositor through the surface of the bean (stinging behavior), and lays an egg on the surface of the larva. Stinging behavior is only observed when females locate beans that contain host larvae.

We previously reported that another parasitic wasp, *Dinarmus basalis* (Rondani) (Hymenoptera: Pteromalidae), utilizes the oviposition-marking pheromone of its host azuki bean weevil as a host-recognizing kairomone (Kumazaki et al., 2000). This prompted us to search for similar kairomone(s) in *A. calandrae*, which paratitizes the same larvae. Here, we report that *A. calandrae* utilizes different constituents of the oviposition-marking pheromone of its host azuki bean weevils as host-recognizing kairomones.

METHODS AND MATERIALS

Host and Wasp. Azuki bean weevils and *A. calandrae* were obtained from colonies maintained at Fujii's laboratory, but which had been subsequently reared for several generations on Chinese green beans, *Vigna radiata* (L.) in Suzuki's laboratory. Although Chinese green beans are smaller than azuki beans (ca. 60 mg/bean), azuki bean weevils developed well in this host. The rearing method of the weevil host and *A. calandrae* is reported elsewhere (Kumazaki et al., 2000). Both sexes of wasps were introduced into plastic Petri dishes (9 cm ID $\times$ 20 mm high) containing Chinese green beans in which host larvae were present (ca. 18 days old). Both insect species were reared at 25°C, 60% relative humidity, and 16L:8D

photoregime. Wasps began to emerge 16–18 days after being exposed to infested beans. The newly emerged wasps (not sexed) were either assayed or used for further reproduction.

Prior to the bioassay, 1- to 3-day-old mated, female wasps were individually released into glass Petri dishes (18 mm ID × 13 mm high; mini P, Tsukuba Flat Co.) on an acrylic plate and conditioned overnight. Only females that showed stinging behavior (2 hr) when presented to a bean with host larvae were bioassayed.

Bioassay. In preliminary studies, the active kairomone(s) eliciting the stinging behavior could be extracted with acetone from beans containing host larvae. In addition, the behavior was observed only when the extract was applied to a fresh bean. Based on these observations, the bioassay in this study was conducted as follows. Two fresh green beans were used for each trial; one was treated with 5 μl [1 mg bean equivalent (BE)] of the test sample, and the other was treated with 5 μl of acetone as a control. After evaporation of the solvent, the beans were placed ca. 1 cm apart on a filter paper (3 × 3 cm) inside an 18-mm glass Petri dish. A mated, 2- to 4-day-old female wasp (described above) was released. Her stinging behavior was observed for 15 min, and the number of stings on each bean was recorded. The assay was repeated 15 times for each sample with a different female. Samples were diluted 10-fold with acetone when necessary. Total number of the stinging behaviors for each sample was compared to the control using Wilcoxon's sign test ($P < 0.05$) (Sokal and Rohlf, 1973).

Analytical Methods. Gas chromatography (GC) was performed with three capillary columns: a fused silica CP-Sil 19 CB column (25 m × 0.25 mm, 0.2 μm; Chrompack, Middelburg, The Netherlands), a heat-resisting MP65HT column (25 m × 0.25 mm, 0.1 μm; Quadrex, New Haven, Connecticut, USA) with a Hewlett-Packard 5890 (Palo Alto, California, USA) gas chromatograph, or a bonded-phase FFAP column (25 m × 0.25 mm, 0.25 μm; Quadrex) with a Hitachi 263-30 gas chromatograph (Hitachi, Ltd., Tokyo, Japan), with nitrogen as the carrier gas. The column temperatures were raised from 100 to 230°C at 4°C/min for analyses of fatty acid methyl esters (FFAP and CP-Sil columns; the injector and detector temperature was set at 250°C) and from 300 to 365°C (held for 10 min) at 5°C/min for analyses of triacylglycerols (MP65HT column; the injector and detector temperature was set at 400°C). Mass spectra were measured with a Hitachi M-80B GC mass spectrometer (MS) coupled with a Hitachi 367-0200 gas chromatograph equipped with an Al-clad methylsilicone capillary column (25 m × 0.22 mm, 0.10 μm, Quadrex) (EI, 70 eV). Mass spectra of methyl esters were obtained by GC-MS (column temperature: 120–270°C programmed at 4°C/min) and those of triacylglycerols were measured by the direct inlet method, using helium as the carrier gas. Thin layer chromatography (TLC) was performed with precoated silica gel $60F_{254}$ plates (E. Merck, Darmstadt, Germany). The developing solvent was a mixture of hexane–ether (7:1, v/v) or hexane–ether–acetic acid (70:30:1, v/v). Spots were detected by exposing the sample to iodine vapor or

spraying with 0.02% rhodamine 6G ethanol solution, followed by UV (365 nm; fluorescent spots). The infrared (IR) spectrum was measured with a JASCO IRA-1 spectrometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan) as a liquid film.

Extraction and Fractionation of Kairomone(s). As described above, the kairomone(s) was (were) extracted with acetone only from beans that had contained host larvae. The selection of beans previously infected by host weevils is important as extracts from uninfected beans were not active. The kairomone(s) was (were) extracted from the green beans (2.6 kg, 30 mg/bean) with acetone (2.5 liters $\times$ 2) at room temperature for two days. The extract was dried, and the major part of the residue (40 of 50 g) was partitioned with ether and water. Half of the active layer (14.8 of 30 g) was chromatographed on a silica gel (SiO_2) column (125 g, 3.5 cm ID $\times$ 37 cm, Wako-gel C-200, Wako, Tokyo, Japan) with a stepwise elution of 1.5 liters each of hexane, hexane–ether (90:10 and then 50:50 v/v), ether, and methanol. Only the 10% ether in hexane fraction (10.8 g) was active, and this was checked by TLC (hexane–ether 7:3, v/v) to give three spots (R_fs $= 0.85$, 0.64, and 0.11), in which the last two spots (the R_f 0.11 spot was tailing) were considered to be triacylglycerols (TGs) and fatty acids (FAs), respectively. The fraction was submitted to Florisil column chromatography (WAK Florisil, Wako) to separate the seven fractions of lipids, according to the method of Carroll (1961). The column (150 g, 3.2 cm ID $\times$ 31 cm) was successively eluted with 1.5 liters each of hexane (fraction 1, 0.04 g), hexane–ether [95:5 (fraction 2, 1.0 g), 85:15 (fraction 3), 75:25 (fraction 4), and 50:50 (fraction 5, 1.2 g)], 2% methanol in ether (fraction 6, 1.6 g), and 4% acetic acid in ether (fraction 7, 3.2 g). Fractions 3 and 4 were combined, because they yielded spots with the same R_f value (0.65) in TLC. This combined fraction (named fraction I, 3.2 g,) and fraction 7 were separately active.

Extraction of Triacylglycerols and Fatty Acids from Chinese Green Beans. Because we used previously infected Chinese green beans, it is possible that the source of the TGs and FAs were derived from green beans. To investigate this possibility, 200 g of crushed fresh green beans were extracted with acetone (200 ml $\times$ 2) for 24 hr. After evaporation of the solvent, the residue was partitioned with ether and water. The ether layer (0.47 g) was submitted to SiO_2 column chromatography (5 g, 10 mm ID $\times$ 14 cm) with stepwise elution of hexane, hexane–ether (90:10, 50:50 v/v), ether, and methanol (50 ml each). TGs and FAs were eluted in 10% ether in hexane (390.7 mg) and analyzed with Florisil column chromatography (5 g, 10 mm ID $\times$ 14 cm). The column was successively eluted with 50 ml each of hexane, hexane–ether (95:5, 85:15, 75:25, and 50:50 v/v), 2% methanol in ether, and 4% acetic acid in ether.

Surface Hexane Washing from Previously Infected Green Beans. Approximately 40 g of green beans were placed into each of five 9-cm plastic Petri dishes, and ca. 150 adult host weevils were added to each dish. After eight days, all of the adult weevils had died and were removed with a sieve, but the hatched larvae had invaded the beans. The surfaces of these beans were washed with hexane for

30 sec, and the TGs and FAs were identified with TLC. The same total volume (200 g) of fresh green beans that had not been invaded by weevils were extracted with hexane for 30 sec as a control.

Synthesis of Triacylglycerols. TGs were synthesized by the following two methods. For method A, commercially obtained 1-monoacylglycerols or 1,3-diacylglycerols (Wako) were treated overnight at 60°C with the desired acyl chlorides in the presence of pyridine. The crude TGs were purified by SiO_2 column chromatography and eluted with 10% ether in hexane. The following nine TGs were prepared (acronyms and yields are given in parentheses): 1-oleoyl-2,3-dipalmitoylglycerol (1O2P3P, 26.4 mg, 94.1%), 1,3-dioleoyl-2-palmitoylglycerol (1O2P3O, 36.7 mg, 89.6%), 1,2-dioleoyl-3-palmitoylglycerol (1O2O3P, 22.1 mg, 76.5%), 1,2-dilinoleoyl-3-palmitoylglycerol (1L2L3P, 10.0 mg, 78.1%), 2-linoleoyl-1-oleoyl-3-palmitoylglycerol (1O2L3P, 4.6 mg, 59.6%), 1,3-dioleoyl-2-stearoylglycerol (1O2S3O, 35.3 mg, 50.0%), 2-linoleoyl-1-oleoyl-3-stearoylglycerol (1O2L3S, 10.4 mg, 73.2%), 2-linoleoyl-1,3-dioleoylglycerol (1O2L3O, 6.9 mg, 65.0%), and 1,2-dilinoleoyl-3-oleoylglycerol (1L2L3O, 4.4 mg, 55.2%).

For method B, three TGs were prepared according to Golding et al. (1993). 2,2-Dimethyl-1,3-dioxolane-4-methanol (100 μl, 0.8 mmol, Wako) was dissolved in 1.5 ml pyridine, to which excess acyl chlorides were added. This was stirred overnight at 60°C. The crude monoacylesters of 2,2-dimethyl-1,3-dioxolane-4-methanol were treated by SiO_2 column chromatography and eluted with 10% ether in hexane to yield pure esters. The acetonides of the esters were cleaved by treating with trifluoroacetic acid in 80% THF aqueous solution and stirring at 0°C in (Leblanc et al., 1986). The crude 1-monoacylglycerols were purified by SiO_2 column chromatography and then reacted with acyl chlorides (two molar equivalents) as described in A: 1-palmitoleoyl-2,3-dipalmitoylglycerol (1P'2P3P, 6.1 mg, 13.4%), 1-linoleoyl-2,3-dipalmitoylglycerol (1L2P3P, 30.3 mg, 17.4%), and 3-oleyl-1,2-distearoylglycerol (1S2S3O, 55.9 mg, 6.2%).

Some acyl chlorides were prepared by the reaction of the desired acids with oxalyl chloride. For example, palmitic acid (20 mg, 0.08 mmol) was dissolved in dry benzene (1 ml), to which equimolar oxalyl chloride was added, and the mixture was refluxed for 1 hr. After benzene and oxalyl chloride were removed, palmitoyl chloride (21.7 mg) was obtained. Palmitoleoyl chloride and stearoyl chloride were prepared in the same way.

RESULTS

Chemical Analysis of Active Fraction 7. Kairomonal activity was found only from fraction 7 (active at 10^{-2} BE) and Fraction I (active at 10^{-1} BE). No synergistic effect was observed between the fractions because their mixture did not

TABLE 1. KAIROMONAL ACTIVITY OF FRACTIONS

Sample	Dose (mg BE)	Total stinging behaviors (N)[a] Sample	Control	P	Activity
Acetone extract	10^{-2}	42	18	<0.025	+
	10^{-3}	42	24	NS	−
Ether layer	10^{-2}	51	8	<0.005	+
	10^{-3}	36	12	NS	−
Aqueous layer	10^{-2}	33	28	NS	−
	10^{-3}	27	47	NS	−
Fractions separated by SiO_2 column chromatography					
H fraction	10^{-1}	23	21	NS	−
10% E/H fraction	10^{-1}	27	1	<0.025	+
	10^{-2}	29	11	NS	−
50% E/H fraction	10^{-1}	12	14	NS	−
E fraction	10^{-1}	15	14	NS	−
MeOH fraction	10^{-1}	12	15	NS	−
Fractions separated by Florisil column chromatography					
Fraction 1	10^{0}	35	29	NS	−
	10^{-1}	16	4	NS	−
Fraction 2	10^{0}	13	13	NS	−
	10^{-1}	12	4	NS	−
Fraction 3 + fraction 4 (fraction I)	10^{-1}	36	4	<0.025	+
	10^{-2}	20	8	NS	−
Fraction 5	10^{0}	7	3	NS	−
	10^{-1}	3	4	NS	−
Fraction 6	10^{0}	5	12	NS	−
	10^{-1}	13	20	NS	−
Fraction 7	10^{-1}	26	9	<0.05	+
	10^{-2}	34	5	<0.025	+
	10^{-3}	19	31	NS	−
Fraction I + fraction 7	10^{-2}	36	12	<0.025	+
	10^{-3}	18	15	NS	−

[a] Total number of stinging behaviors for each bean during 15 min ($N = 15$). BE = bean equivalent; NS: not significant; H: hexane; E: ether; MeOH: methanol; AcOH: acetic acid. +: active; −: inactive.

enhance the activity (Table 1). In view of their TLC (hexane–ether 7:3, v/v) and Florisil column chromatographic behaviors, the chemicals were considered to be FAs ($R_f = 0.13$, tailing) and TGs ($R_f = 0.65$), respectively.

Fraction 7 was treated with diazomethane to yield methyl esters, which were analyzed by GC and GC-MS and compared with the authentic samples. Ten peaks were detected and identified (Table 2). The fraction 7 (3.2 g) obtained from 1.04 kg green beans, with emerged weevil removed, had a 10^{-2} BE (active dose) of 30.9 ng FAs. Thus, the absolute amount of each acid in 10^{-2} BE of fraction 7 was calculated on the basis of the GC peaks (Table 2). The most abundant acid was palmitic acid (40.8%). Kairomonal activity of authentic acids or their mixtures was assayed

TABLE 2. FATTY ACID COMPOSITION OF ACTIVE FRACTION

Fatty acid	Composition (%)	Absolute amount (ng)[a]
Lauric acid	0.1	0.03
Myristic acid	0.8	0.25
Pentadecanoic acid	0.1	0.03
Palmitic acid	40.8	12.61
Palmitoleic acid	0.7	0.22
Heptadecanoic acid	0.3	0.09
Stearic acid	6.7	2.07
Oleic acid	33.0	10.20
Linoleic acid	12.7	3.92
Linolenic acid	4.8	1.48

[a] Absolute amount in active dose (10^{-2} mg BE, 30.9 ng). BE = bean equivalent.

at doses of 10^0–10^{-3} BE (Table 3). Individual acids, or a mixture of five major acids, were inactive, but the complete mixture (10 acids) was active at a dose of 10^{-2} BE (natural ratio), whose activity was equal to those of the acetone extract and fraction 7.

TABLE 3. KAIROMONAL ACTIVITY OF AUTHENTIC ACIDS

Sample	Dose (mg BE)	Stinging behaviors (N)[a]		P	Activity
		Sample	Control		
Mixture of 10 acids[b]	10^{-1}	46	10	<0.025	+
	10^{-2}	25	9	<0.05	+
	10^{-3}	18	14	NS	–
Mixture of 5 acids[c]	10^{0}	6	5	NS	–
	10^{-1}	16	10	NS	–
	10^{-2}	4	15	NS	–
Lauric acid	10^{-1}	44	25	NS	–
Myristic acid	10^{-1}	20	11	NS	–
Pentadecanoic acid	10^{-1}	16	11	NS	–
Palmitic acid	10^{-1}	9	12	NS	–
Palmitoleic acid	10^{-1}	22	11	NS	–
Heptadecanoic acid	10^{-1}	26	29	NS	–
Stearic acid	10^{-1}	28	22	NS	–
Oleic acid	10^{-1}	14	13	NS	–
Linoleic acid	10^{-1}	14	11	NS	–
Linolenic acid	10^{-1}	24	16	NS	–

[a] Total no. of stinging behaviors for each sample during 15 min ($N = 15$). NS: not significant; +: active; –: inactive. BE = bean equivalent.
[b] A mixture of 10 acids (natural ratio).
[c] A mixture of palmitic, stearic, oleic, linoleic, and linolenic acids (natural ratio).

Chemical Analysis of Active Fraction I. Another active fraction (Fraction I) was comprised of TGs. Its IR spectrum showed the following frequencies: 2940, 2860, 1750 (ester C=O), 1470, and 1160 cm^{-1}. No OH stretching frequency was observed. In TLC, fraction I gave a spot with $R_f = 0.64$ (trioleoylglycerol, $R_f = 0.64$) (hexane–ether 7:3, v/v).

Fraction I showed the activity at a dose of 10^{-1} BE, which contained 309 ng of TGs. An aliquot of fraction I was hydrolyzed with KOH in ethanol–water overnight at room temperature. The acidic fraction was analyzed by GC and GC-MS, after methylation with diazomethane. The GC profile was very similar to that of the methyl esters of fraction 7. The aqueous layer, upon evaporation of ethanol and water after neutralization, was heated with potassium hydrogen sulfate ($KHSO_4$) to confirm the presence of glycerol, using the acrolein test (Hayashi, 1991). The characteristic pungent odor of acrolein confirmed the presence of glycerol.

The MS spectrum of the TGs (fraction I) was measured with the direct inlet method, and the following fragments were detected; *m/z* 57 (base peak), 237, 239, 261, 263, 265, 267 (fatty acid residues), 336, 338, 340, 549, 551, 571, 573, 575, 579, 601, 603, and 605 (residues leaving FAs from TGs) (Murata and Takahashi, 1973).

Direct GC analysis of the TGs involved in fraction I was conducted using an MP65HT capillary column. The column temperature was 300–360°C programmed at 5°C/min. A total of 13 peaks were observed from the gas chromatogram (Figure 1), which were separated on the basis of total number of carbons that constituted the FAs of the TGs. They were C_{48} (2.5%), C_{50} (33.1%), C_{52} (50.0%), and C_{54} (14.4%), compared with those of the synthetic TGs (Table 4). The major components of the TGs were PPO (24.2%) and POO (26.3%). In this column, TGs with the same total number of carbons as those of FAs, but with different carbon positions, could not be distinguished. For example, 1,2-dipalmitoyl-3-oleoylglycerol (1P2P3O), and 1,3-dipalmitoyl-2-oleoylglycerol (1P2O3P) yielded one peak.

TGs and FAs from Chinese Green Beans. Florisil column chromatography separated 153 mg TGs and 124.1 mg FAs from 5 and 15% ether in hexane and 4% acetic acid in ether fractions, respectively. These fractions were obtained from 200 g (ca. 60 mg/bean) of crushed green beans and were calculated as 45.9 μg TGs and 37.3 μg FAs per bean, or 765 μg TGs and 620.5 μg FAs per g beans.

TGs and FAs in Surface Hexane Washings of Green Beans from Which Weevils Were Removed. The hexane washing from the beans oviposited by weevils produced 72.5 mg or 38.5 μg/bean of hydrocarbons (the main component), TGs and FAs, which were identified with TLC (hexane–ether 7:3, v/v). Conversely, the hexane washing from the fresh bean control (no weevil oviposition) produced almost no residue (0.3 mg).

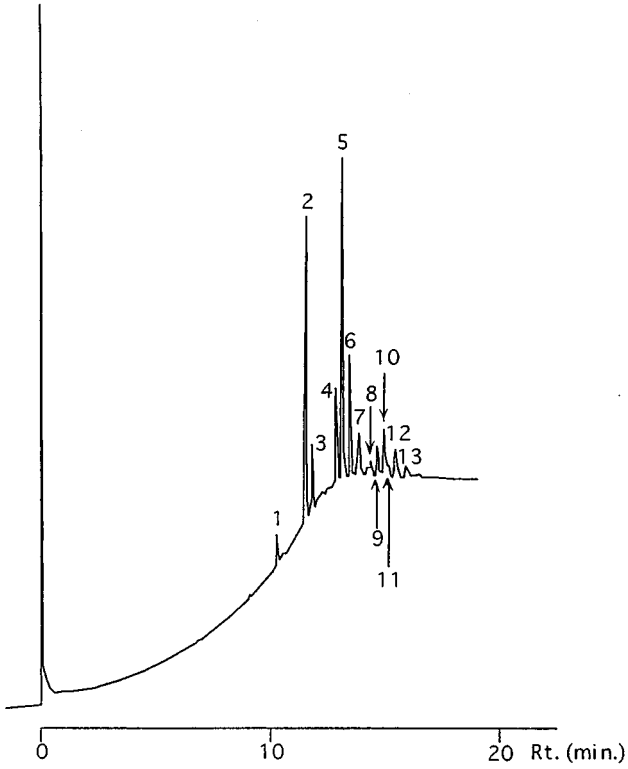


FIG. 1. Gas chromatogram of the active triacylglycerols (fraction I). Condition: MP65HT capillary column; 300–365°C (10 min) at 5°C/min.

DISCUSSION

The host-recognizing kairomones for the parasitic wasp, *A. calandrae*, were demonstrated to be comprised of fatty acids (FAs) and triacylglycerols (TGs). Both fractions separately elicited kairomonal activity, but no synergistic effect was observed. The activity of the FAs (fraction 7) was more than 10^{-2} mg BE (30.9 ng), whereas for the TGs (Fraction I) it was more than 10^{-1} mg BE (309 ng). Thus, fraction 7 was ten times more active than Fraction I. Fraction 7 was composed of 10 carboxylic acids, of which palmitic acid was main component (40.8%). Individual acids or a mixture of five major acids were inactive, but the complete acid mixture was active. This suggests that minor acids were required for activity.

The FAs constituting the TGs in fraction I were characterized, although their attaching positions to glycerol were not determined. The carbon numbers

TABLE 4. TOTAL CARBON NUMBERS OF FATTY ACIDS AND THEIR CONSTITUENTS, AND COMPOSITION OF EACH TRIACYLGLYCEROL INVOLVED IN FRACTION I

GC peak no.[a]	Total carbon no. of acid constituents	Acid constituent	Composition (%)
1	C_{48}	PPP'	2.5
		Subtotal	2.5
2	C_{50}	PPO	24.2
3		PPL	8.9
		Subtotal	33.1
4	C_{52}	PSO	10.6
5		POO	26.3
6		POL	10.1
7		PLL	3.0
		Subtotal	50.0
8	C_{54}	SSO	1.5
9		SOO	2.7
10		OOO	4.2
11		SOL	0.9
12		OOL	3.5
13		OLL	1.6
		Subtotal	14.4

[a] GC peak no. corresponds that of Figure 1. P: palmitic; P': palmitoleic; S: stearic; O: oleic; L: linoleic acids.

constituting the FA component of the TGs in this fraction ranged from C_{48} to C_{54}, in which C_{52} (50.0%) was the major components.

Azuki bean weevils secrete an oviposition-marking pheromone to prevent multiple oviposition for a bean, which consists of FAs, hydrocarbons, and TGs (Oshima et al., 1973; Sakai et al., 1986; Honda and Ohsawa, 1990) in a ratio of ca. 1:4.7:2. Of these three constituents, the FA fraction is composed of palmitic (21.6%), stearic (11.5%), oleic (45.5%), linoleic (10.7%), and linolenic (4.1%) acids and 7.4% other acids; the TG fraction comprises a mixture of C_{48} (1.0%), C_{50} (18.9%), C_{52} (47.7%), C_{54} (31.7%), and C_{56} (0.4%) (Honda and Ohsawa, 1990). In the present study, the active kairomones for *A. calandrae* were also comprised of FAs and TGs and are almost the same constituents as for those of the host pheromone. Although TGs and FAs were components of the control fresh green beans (765 and 620.5 μg/bean, respectively), the major fractions of the separated kairomones are thought to be insect-derived, because the amount of TGs and FAs obtained from previously infected green beans was calculated to be 3120 μg/g

beans, i.e., ca. 4–5 times more than those obtained from the control beans. In addition, hexane washing of surface green beans yielded almost no residue. Thus, the major part of the kairomones must be derived from the host pheromone.

We previously reported that other pteromalid wasp, *Dinarmus basalis*, utilizes the saturated hydrocarbons (and diacylglycerols) of the oviposition-marking pheromone of azuki bean weevils as a host-recognizing kairomone (Kumazaki et al., 2000). Thus, the two pteromalid wasps selectively utilize different constituents of the oviposition-marking pheromone of azuki bean weevils as host-recognizing kairomones. For *D. basalis*, TGs as well as diacylglycerols were active. Moreover, both saturated hydrocarbons and diacylglycerols (triacylglycerols) were required for activity in this wasp, whereas in *A. calandrae*, FAs and TGs were separately active, and no synergistic effect was observed between them. *D. basalis* mainly parasitizes azuki bean weevils, whereas *A. calandrae* also attacks other bruchid species. The narrower host range of *D. basalis* compared to *A. calandrae* might be because both constituents of its host pheromone are necessary to search for, or recognize, its host larvae. It would be interesting to know whether FAs and TGs can independently promote stinging behavior.

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CUTICULAR HYDROCARBON PROFILES OF DOMINANT VERSUS SUBORDINATE MALE *Nauphoeta cinerea* COCKROACHES

ESTELLE ROUX,[1] LEAM SRENG,[1,3,*] ERIC PROVOST,[1,4] MAURICE ROUX,[2] and JEAN-LUC CLEMENT[1]

[1]*CNRS, Laboratoire. de Neurobiologie "Communication Chimique"*
31, Chemin Joseph Aiguier
13402 Marseille Cedex 20, France

[2]*Université d'Aix-Marseille III*
Laboratoire de Biomathématiques
Av. Escadrille Normandie Niemen
13397 Marseille, France

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Abstract—Interactions between male *Nauphoeta cinerea* cockroaches are characterized by an elaborate ritual that leads to a stable dominant–subordinate hierarchy between two individuals. Chemical signals involving volatile sex pheromones and cuticular hydrocarbons play an important role in establishing and maintaining dominance status. The present study was performed to identify cuticular hydrocarbons in two- and three-times dominant or subordinate individuals obtained by forcing dyadic encounters. Two methods, i.e., solid-phase microextraction (SPME) and organic solvent extraction, were used to isolate cuticular hydrocarbons. A total of 23 peaks of cuticular hydrocarbons were identified. Analysis showed quantitative differences in hydrocarbon profiles of three-times dominant and subordinate individuals according to extraction method and dominance status. Dominant individuals were characterized by higher proportions of the monomethylalkanes 11- and 13-MeC_{36}, 13- and 15-MeC_{38}, and 11-, 13-, and 15-MeC_{35}, while subordinate individuals had higher proportions of the following monomethylalkanes: 7-, 9-, and 11-MeC_{31}, 5-MeC_{31}, 3- and 8-MeC_{32}, and 9-, 10- , 11-, and 12-MeC_{32}. Discussion focuses on the reliability of hydrocarbons as indicators of dominance status and on their correlation with physiological processes.

* To whom correspondence should be addressed. E-mail: sreng@irlnb.cnrs-mrs.fr

[3] Present address: CNRS, Institut de Neurosciences Physiologiques et Cognitives (INPC), 31, Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France.

[4] Present address: CNRS, Institut Méditerranéen d'Ecologie et de Paléoécologie (IMEP), Europôle Méditerranéen de l'Arbois, 13090 Aix en Provence, France.

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Key Words—Dominance status, Hydrocarbon profiles, SPME, cockroaches, *Nauphoeta cinerea*.

INTRODUCTION

Social stability in many orders of insects depends on the outcome of agonistic encounters that confer dominant individuals with special rights with regard to water, food, shelter, and mating. Numerous studies have concentrated on the elaborate agonistic interactions between mature male *Nauphoeta cinerea* cockroaches (Ewing, 1967, 1972, 1973; Ewing and Ewing, 1973; Bell et al., 1979; Bell and Gorton, 1978; Breed et al., 1980; Schal and Bell, 1983). Depending on population density, these encounters determine either hierarchy (high density) or territoriality (low density). Various interrelated aspects of this behavior have been studied, including the role of volatile sex pheromones, importance of posture, and physiological impact of dominance versus subordination on physiological activity.

Based on early reports by Ewing (1967) indicating that dominant males respond to subordinates before making contact, it was speculated that volatile signals are involved in these interactions. After more than 30 years of study, a great deal of data has been gathered on volatile sex pheromone in male cockroaches (Sreng, 1990; Sirugue et al., 1992; Moore et al., 1995). The presence of a sex pheromone called "seducin" was first demonstrated in the abdominal region of male *N. cinerea* in 1966 by Roth and Dateo (1966), but its origin from the sternal glands was not elucidated until 1979 (Sreng, 1979), and its exact chemical composition was not determined until 1990 (Sreng, 1990). Various studies have shown that sex pheromone plays a fundamental role in male–male agonistic behavior and dominance status (Breed et al., 1980; Smith and Breed, 1982; Schal and Bell, 1983; Moore, 1988, 1989, 1997; Sreng, 1991; Moore et al., 1995, 1997). The great variability in the proportion of the three components identified in the sex pheromone (Sreng, 1990) suggests that each male has a characteristic blend (personal odor) depending on its status in the group. However, in a detailed study of ontogenetic and social factors involving paired comparison of the ratios of the three sex pheromone components, Moore et al. (1995) showed that only the ratio between 2-methylthiazolidine and 3-hydroxy-2-butanone differed significantly between subordinate and dominant males. The study of volatile sex pheromones is obviously subject to chemical and physical limitations. Another factor to consider is the effect of male status on the level of biosynthesis activity in glandular cells and on storage in glandular epithelium or reservoir (Sreng, 1985).

Behavioral studies have shown that male posture plays an important role in mating competition between male cockroaches. Since cockroaches live in darkness, vision plays little role in the perception of posture. This was confirmed by Schal and Bell (1983) by eye-painting experiments. In a report describing the discovery

of male abdominal glands, Sreng (1979) also noted that males assumed a special stance, or calling posture, when releasing sex pheromone produced by the sternal glands. Release of sex pheromone can be enhanced by expanding the abdomen to expose the glandular orifices situated at the anterior portion of abdominal segments (Sreng, 1979, 1984, 1985, 1990, 1991, 1993). This position, also termed the aggressive posture (Ewing, 1967, 1972; Bell and Gorton, 1978), is normally associated with dominant males (Ewing, 1967; Moore et al., 1995) and is adopted immediately after winning an encounter. Only dominant males display the calling posture releasing sex pheromone that determines access to receptive females and mating success (Breed et al., 1980; Schal and Bell, 1983; Sreng, 1991; Moore et al., 1995). The same posture has been observed in the same context in many species of Oxyhaloinae (Fraser and Nelson, 1984; Leibensperger et al., 1985; Sreng 1993; Clark and Moore, 1995).

Physiological correlates of agonistic behavior in male cockroaches have received less attention than sex pheromone or posture. In one study, it was shown that stress induced by domination could cause death in subordinate males (Ewing, 1967). Electrophysiological recordings showing high ventral nerve cord activity in subordinate males confirmed that agonistic behavior led to profound physiological changes (Ewing and Ewing, 1973). Several authors demonstrated that physiological changes probably involve sex pheromone (Moore et al., 1995) as well as cuticular hydrocarbons. Previous studies in a variety of species have demonstrated that the proportion of cuticular hydrocarbons depends on physiological status [Arnold and Régnier (1975) in flies; Antony and Jallon (1981) and Jallon et al. (1982) in drosophila; Bonavita-Cougourdan et al. (1991) in wasps; and Schal et al. (1991) and Everaerts et al. (1997) in cockroaches].

The main purpose of this study was to identify cuticular hydrocarbons in male *Nauphoeta cinerea* cockroaches and determine their correlation with dominant versus subordinate status. Secondarily, we compared the efficiency of conventional solvent extraction and solid-phase microextraction (SPME) for cuticular hydrocarbon extraction.

METHODS AND MATERIALS

Animals. The cockroaches used in this study were obtained from mixed-age and mixed-sex colonies of *Nauphoeta cinerea* (Blaberidae, Oxyhaloinae) reared in an aquarium ($60 \times 40 \times 20$ cm) at $26 \pm 1^{\circ}$C and 70% relative humidity with a reversed 12L:12D photoperiod. Insects were allowed free access to dry dog food and water. After removal from the colony, newly emerged males were isolated in round plastic boxes (10 cm diam.) pending behavioral tests at the age of 12 days. To enable identification during behavioral tests, a number tag was attached with an adhesive used by beekeepers to mark queens. Tagging was performed on the pronotum of each animal to avoid contamination during extraction.

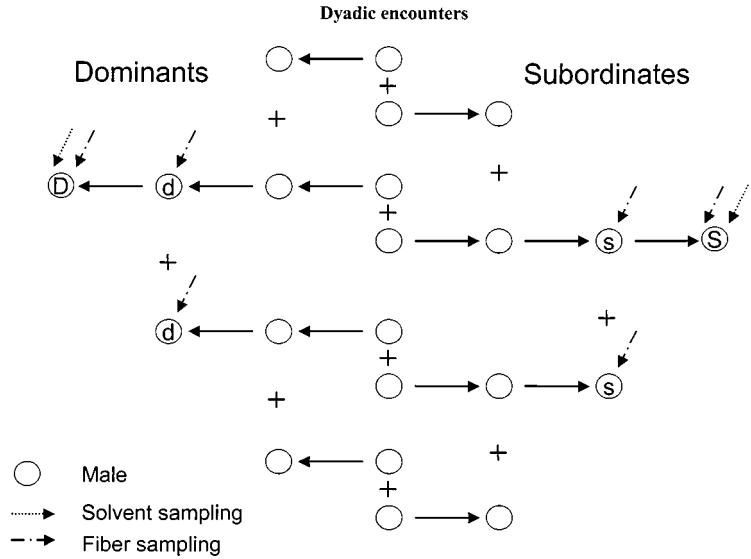


FIG. 1. Behavioral experiment used to obtain two-times dominant (d) or subordinate (s) individuals and three-times dominant (D) or subordinate (S) individuals by forcing dyadic encounters. Arrows indicate the sampling techniques used.

Behavioral Tests. Dyadic agonistic encounters were carried out as follows: In the first test, two untried males were placed in a new plastic dish. Hierarchy was established by observing the outcome of the encounter. The male that continued to move about the dish with its body high off the ground and abdomen extended was declared the winner (dominant), while the male that ran away and assumed the crouched rocklike posture with its legs folded up and antenna on the ground was declared the loser (subordinate) (cf. Ewing, 1967). In the second and third tests, winners were confronted with winners and losers with losers. In this way, we obtained two-times and three-times winners and losers, i.e., two-times dominant (d), two-times subordinate (s), three-times dominant (D), or three-times subordinate (S) (Figure 1).

Extraction of Cuticular Hydrocarbons. Hydrocarbons were sampled using solid phase microextraction (SPME) and pentane extraction (Figure 1). Since SPME involves solventless recovery and concentration of substances on silica fibers coated with various stationary phases, it can be repeated more than once on the same animal. In this study, SPME was used twice, i.e., once on two-times dominant and subordinate males and once on three-times dominant and subordinate males. In contrast, since pentane solvent extraction requires destruction of the specimen, it was used only on three-times dominant animals.

SPME was performed using a Supelco 5-7308 fiber (polydimethylsiloxane phase, 30 μm thickness). During sampling, cockroaches were anesthetized with carbon dioxide and restrained with five staples placed over the body of the animal in order to expose the usually hidden glandular part of the third sternite (Sreng, 1984). Care was taken not to damage the animal physically. After cleaning by deabsorption in the GC injector at 250°C for 30 min, the fiber was rubbed gently against the sternal gland for 1 min. Similar SPME techniques have been used to study pheromone production in ants and weevils (Malosse et al., 1995; Monnin et al., 1998).

To enhance comparability with SPME, which involved only the sternite 3, pentane extraction was performed on a fragment of abdominal cuticle removed with microsurgical scissors. After wiping off adjacent tissue (hemolymph and fat body) with a paper towel, the cuticule fragment was placed in pentane for 20 min at room temperature. The resulting extracts were stored at −20°C pending analysis. Prior to analysis extracts were dried under a nitrogen stream in a conical vial and resuspended in 10 μl of pentane.

Chemical Analysis. SPME samples and pentane extracts were analyzed by gas chromatography (GC). The chromatograph was a Delsi Nermag DN 200 equipped with a flame ionization detector, split/splitless injector (splitless mode, 15 sec), and a capillary column (Chrompack CPSil. 5 WCOT: 25 m long × 0.25 mm diam., 0.25 μm). It was coupled with an Enica 31 integrator. Oven temperature was programmed from 150°C for 5 min to 250°C at 10°C/min, then to 320°C at 5°C/min (final time, 15 min). The carrier gas was helium at 1 bar. SPME samples were injected by desorbing the fiber in the injection port for 15 min at 250°C. Individual insect pentane extracts (2 μl) were injected in the chromatograph.

Hydrocarbons were identified by gas chromatography–mass spectrometry (GC-MS), performed on a Hewlett Packard 5890 coupled with a 5989A mass spectrometer. The capillary column and temperature program were the same as those used for GC. The system was programmed with a HPUX MS Chemstation controller. Analyses were performed in the electron impact mode (EI, 70 eV) and chemical ionization mode (CI, methane).

Data Analysis. Twenty-three peaks, representing a single component or mixture of components, were selected for analysis. Any peak causing integration problems due to background noise was discarded. Compounds with similar retention times were combined. As described by Bagnères et al. (1991), peak areas given by the integrator were corrected as a function of chain length using the following correction factor: $K = 0.042X + 0.11$, where X is the number of carbon atoms per molecule of the substance. Statistical analyses were conducted on the proportion of each peak in relation to the overall number of peaks obtained in each specimen as calculated with a Microsoft Excel 5 spreadsheet.

Comparison of SPME and pentane extraction was carried out on data from eight three-times dominant or subordinate individuals (set A). Principal component

analysis (PCA) was performed by using the Le Statoscope software package. The most representative hydrocarbons on factorial axes, i.e., those for which the squared values of the correlation coefficient between the variable and axes were from 0.65 to 1, were chosen for study. Hydrocarbon selection was also assisted by the Wilcoxon signed rank test (for paired samples).

To further analyze the correlation between hierarchy status and cuticular hydrocarbon profile, a second PCA was carried out using corrected data from a table designed to eliminate the possible effects of extraction technique on the dispersion of the individuals on the factorial plane. The correction table was constructed as follows. For each and every compound (j), we compared its mean value for fiber extraction (F), let it be $m_F(j)$, and its mean value for the eight pentane extractions (P), let it be $m_P(j)$. Each x_j value was then replaced by [$(x_j - m_F(j))$ or $(x_j - m_P(j))$], depending on which extraction technique had been used. A Mann-Whitney U test was carried out on the factorial coordinates of the first axis of this PCA. To assess the role of compounds, we performed the Friedman nonparametric test, with extraction mode as blocks and dominance status as factors.

Another PCA, using hydrocarbon data from 29 SPME (set B), was performed to confirm the correlation between cuticular profile and dominance status. Eight of the 29 tests involved the same animal tested after the second and third encounters, i.e., when they were two- and three-times dominant or subordinate (Figure 1). A Kruskal-Wallis test with the factorial coordinates was carried out to clarify the distribution of individuals according to profile on the first axis. Mean values for each status were introduced in the PCA as supplementary variables and encoded as follows: DOM = three-times dominant individuals, dom = two-times dominant individuals, SUB = three-times subordinate individuals, sub = two-times subordinate individuals. Again, to assess the role of each compound the Kruskal-Wallis test was individually applied to all compounds.

RESULTS

Chemical Analysis. The 23 peaks identified in *Nauphoeta cinerea* cockroaches contained a variety of hydrocarbons, including normal alkanes from C_{25} to C_{33}, monomethylalkanes from C_{26} to C_{39}, dimethylalkanes from C_{34} to C_{37}, and one trimethylalkane of C_{36} (Figure 2, Table 1).

Comparison of Extraction Techniques. PCA using the eight three-times dominant or subordinate individuals (set A), using both SPME and solvent extraction, demonstrated a clear-cut difference between the two techniques. Individuals were separated along a linear combination (axis δ) of the first and second axes, accounting for 59.4% of total variance (Figure 3a). Pentane extracts projected on the positive side of axis δ, whereas all SPME but one (rDF) projected on the negative side. Pentane extracts were characterized by a higher proportion of cholesterol (cholest-5-en-3-ol, peak s5) and n-C_{33} (peak a13) (Figure 3b, upper right area),

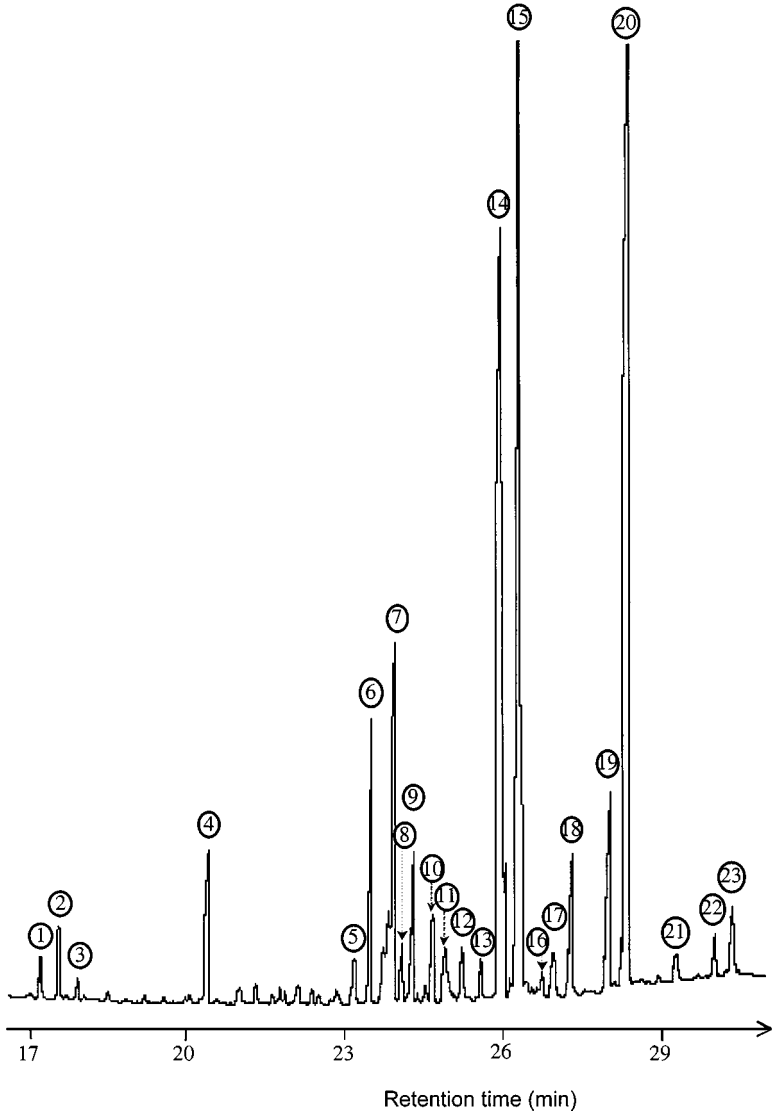


FIG. 2. Gas chromatogram of the cuticular hydrocarbons of a dominant male. Each peak corresponds to one compound or mixture of several compounds listed Table 1.

whereas SPME samples were characterized by a higher proportion of monomethylalkanes with C_{25} (13-MeC_{25}, peak m2), C_{31} (7-, 9-, 11-MeC_{31}, peak m7, 5-MeC_{31}, peak m8), C_{32} (3-, 8-MeC_{32}, peak m10) and dimethylalkanes with C_{33} (11-, 21-DimeC_{33}, peak d15) (Figure 3b, lower left area). According to the Wilcoxon signed

TABLE 1. CUTICULAR HYDROCARBONS FROM *Nauphoeta cinerea*[a]

Peak	Compound	Mol wt	Diagnostic ions	$[M-H]^+$
a1	*n*-C_{25}	352	352	351
m2	13-MeC_{25}	366	196/197	365
m3	3-MeC_{25}	366	56/57, 336/337	365
a4	*n*-C_{29}	408	408	407
s5	Cholest-5-en-3-ol	386	107, 145, 159, 213, 275, 301, 353, 368, 386	
a6	*n*-C_{31}	436	436	435
m7	7-; 9-; 11-MeC_{31}	450	112/113, 140/141, 168/169, 308/309, 336/337 364/365	449
m8	5-MeC_{31}	450	84/85, 292/293	449
m9	3-MeC_{31}	450	56/57, 420/421	449
a9'	*n*-C_{32}	450	450	449
m10	3-; 8-MeC_{32}	464	56/57,126/127, 364/365, 434/435	463
m11	9-; 10-; 11-; 12-MeC_{32}	464	140/141, 154/155, 168/169, 182/183, 308/309, 322/323, 350/351, 336/337	463
d12	10,16-; 10,20-DimeC_{32}	478	154/155, 196/197, 252/253, 308/309, 350/351,	477
a13	*n*-C_{33}	464	464	463
m14	9-; 11- or 23-; 13-MeC_{33}	478	140/141, 168/169, 196/197, 308/309, 336/337, 364/365	477
d15	11, 21-DimeC_{33}	492	168/169, 196/197, 322/323, 350/351, 477	491
d16	3, 7-; 3, 9-DimeC_{33}	492	56/57, 126/127, 154/155, 364/365, 392/393, 462/463	491
m17	11-MeC_{34}	492	168/169, 350/351	491
t18	11, 15, 21-TrimeC_{33}	506	168/169, 196/197, 238/239, 294/295, 336/337, 364/365, 491	505
m19	11-; 13-; 15-MeC_{35}	506	168/169, 196/197, 224/225, 308/309, 336/337, 364/365	505
m20	11-; 13-MeC_{36}	520	168/169, 196/197, 350/351, 378/379	519
d21	8, 15-; 10, 17-DimeC_{35}	520	126/127, 154/155, 238/239, 266/267, 280/281, 308/309, 392/393	519
m22	13-; 23-MeC_{37}	534	196/197, 224/225, 336/337, 364/365	533
m23	13-; 15-MeC_{38}	548	196/197, 224/225, 350/351, 378/379	547

[a] Abbreviations: alkanes, a; monomethylalkanes, m; dimethylalkanes, d; trimethylalkane, t; cholesterol, s.

rank test, peaks a1, a4, m7, m8, m10, and d12 were discriminant at the 5% level, and peaks m2, m3, s5, a13, and d15 were discriminant at the 1% level.

Correlation between Status and Hydrocarbon Profile. A second PCA was performed after correction of set A data to eliminate the effect of extraction technique (Figure 4a). Dominant individuals (negative side) and subordinate individuals (positive side) were separated on the first axis in all but three cases (uSP, kDF, qDF, for nomenclature see legend). A Mann-Whitney test with the factorial coordinates on the first axis confirmed nonrandom separation ($z = 59$, $P < 0.05$). Dominant

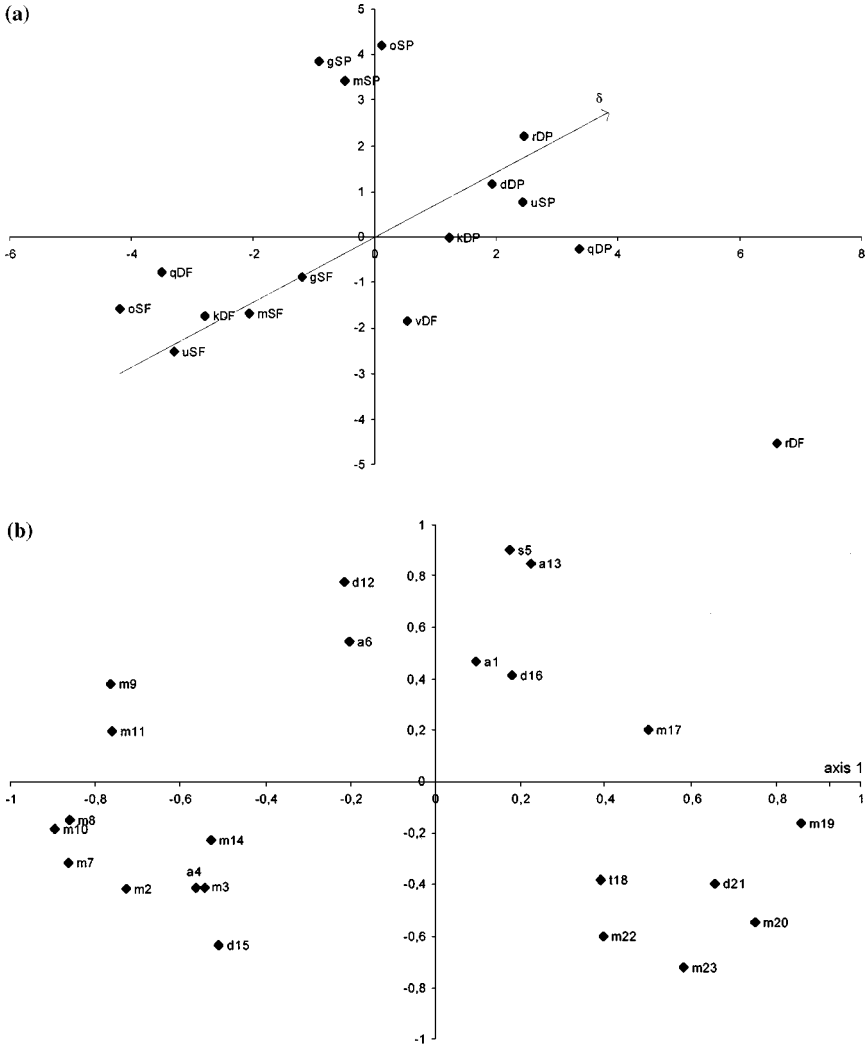


FIG. 3. (a) Principal component analysis of 23 variables (proportions of hydrocarbons) from eight three-times dominant individuals and eight three-times subordinate individuals (set A, $N = 16$). Projection was performed on factorial plane 1–2. Axis 1 accounts for 34.7% of total variance and axis 2 for 24.7%. Axis δ discriminates the two types of individuals. Solvent (xxP) and fiber (xxF) samples from three times dominants (xDx) or subordinate (xSx) individuals were compared. The letter in front of the status designates the individual insect. (b) Projection of the hydrocarbons on axes 1 and 2. Abbreviations: alkanes, a; monomethylalkanes, m; dimethylalkanes, d; trimethylalkane, t; cholesterol, s.

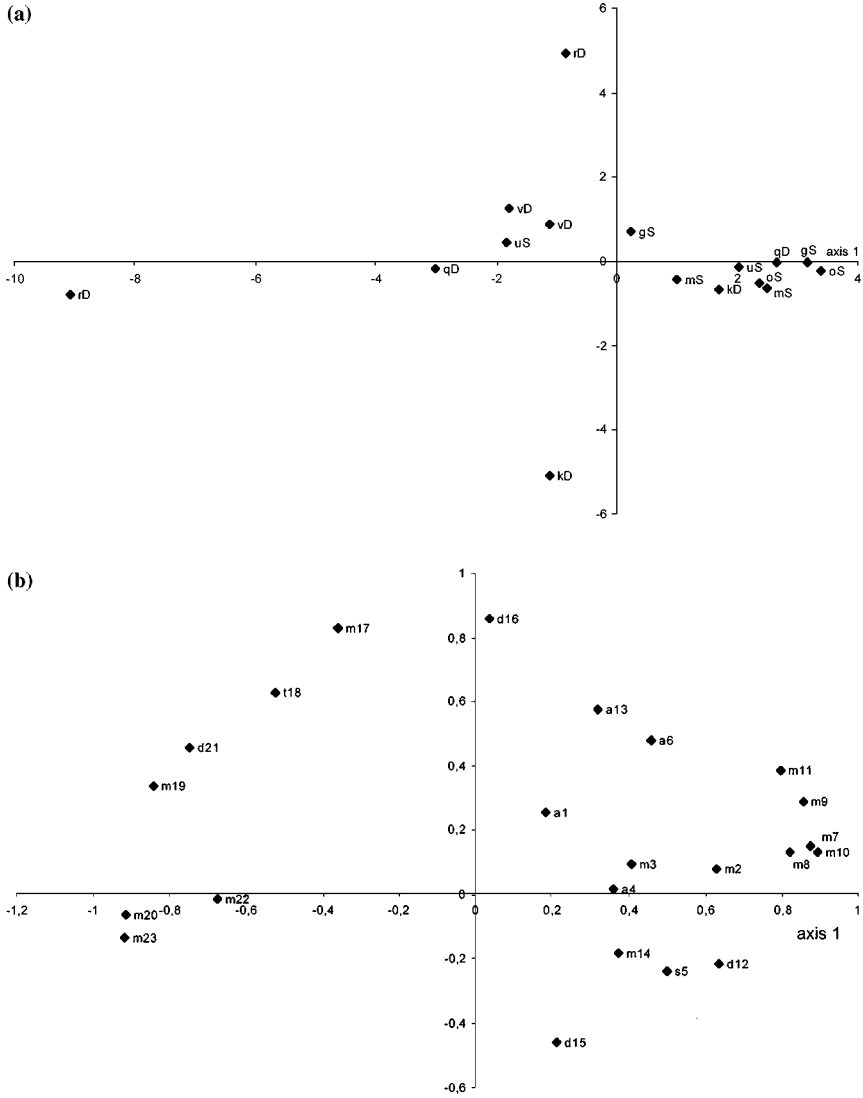


FIG. 4. (a) Principal component analysis of 23 variables (proportions of hydrocarbons) using corrected data from the same eight three-times dominant individuals and eight three-times subordinate individuals as in Figure 3 (corrected set A, $N = 16$). Projection was performed on factorial plane 1–2. Axis 1 accounted for 40.4% of total variance and axis 2 for 15.0%. Dominance status of three times dominant (xD) or subordinate (xS) individuals were compared. The letter in front of the status designates the individual insect. (b) Projection of the hydrocarbons on axes 1 and 2. Abbreviations as in Figure 3.

individuals were characterized by a higher proportion of monomethylalkanes: 11-, 13-, 15-MeC_{35}, peak m19; 11-, 13-MeC_{36}, peak m20; and 13-, 15-MeC_{38}, peak m23; whereas subordinate individuals were characterized by a higher proportion of monomethylalkanes: 7-, 9-, 11-MeC_{31}, peak m7; 3-MeC_{31}, peak m9; 3-, 8-MeC_{32}, peak m10; and 9-, 10-, 11-, 12-MeC_{32}, peak m11 (Figure 4b). The Friedman nonparametric test indicated that peaks m2, s5, and m9 were discriminant for dominance status at the 5% level.

The PCA performed on set B data corresponding to tests on two- and three-times dominant or subordinate individuals also showed a significant difference between dominant and subordinate individuals. As shown in Figure 5a, the four groups (D, d, S, s) lined up in their logical order on the first factorial axis accounting for 56.20% of total variance. Three-times dominant individuals were in the most negative position. Despite the four exceptions (ls, vD, kD, qD), the Kruskal-Wallis test indicated nonrandom distribution of individuals ($K = 8.27$, $P < 0.05$). This finding was confirmed when dominance level (DOM, dom, sub, SUB) was projected as supplementary variables (Figure 5b). The compounds characterizing dominant individuals were the same as in the previous PCA plus a mixture of monomethylalkanes (11-, 13-, 15-MeC_{35}, peak m19). In contrast, subordinate individuals were characterized by four mixtures involving 7-, 9-, 11-MeC_{31} (peak m7) and 3-, 8-MeC_{32} (peak m10) as previously described and monomethylalkanes 5-MeC_{31} (peak m8) and 9-, 10-, 11-, 12-MeC_{32} (peak m11). The Kruskal-Wallis test showed that peaks m7, m11, and m14 were discriminant at the 5% level.

DISCUSSION

Qualitatively, the cuticular hydrocarbon profiles obtained in dominant and subordinate males using SPME and pentane extraction were identical. Both methods extracted a total of 23 peaks. Differences between SPME and pentane extraction profiles were observed with regard to the proportion of some peaks compounds. Pentane extracted more cholesterol, peak s5, and *n*-alkane *n*-C_{33}, peak a13, whereas SPME extracted more monomethylalkanes 13-MeC_{25}, peak m2; 7-, 9-, 11-MeC_{31}, peak m7; 5-MeC_{31}, peak m8; 3-, 8-MeC_{32}, peak m10; and dimethylalkane 11, 21-$DimeC_{33}$, peak d15. These differences could be due to chemical affinities of the fiber and pentane as observed even when solvents with the similar polarity are used.

Quantitative differences in the hydrocarbon profiles could also be due to the methodological differences between the two extraction techniques. Unlike the solvent, the fiber is applied directly to the target and, thus, all compounds obtained came from the sternal gland. There was no contamination by adjacent tissue. Residual tissue (e.g., fat, hemolymph, and muscle) clinging to the sample after dissection for solvent extraction may be a source of cholesterol.

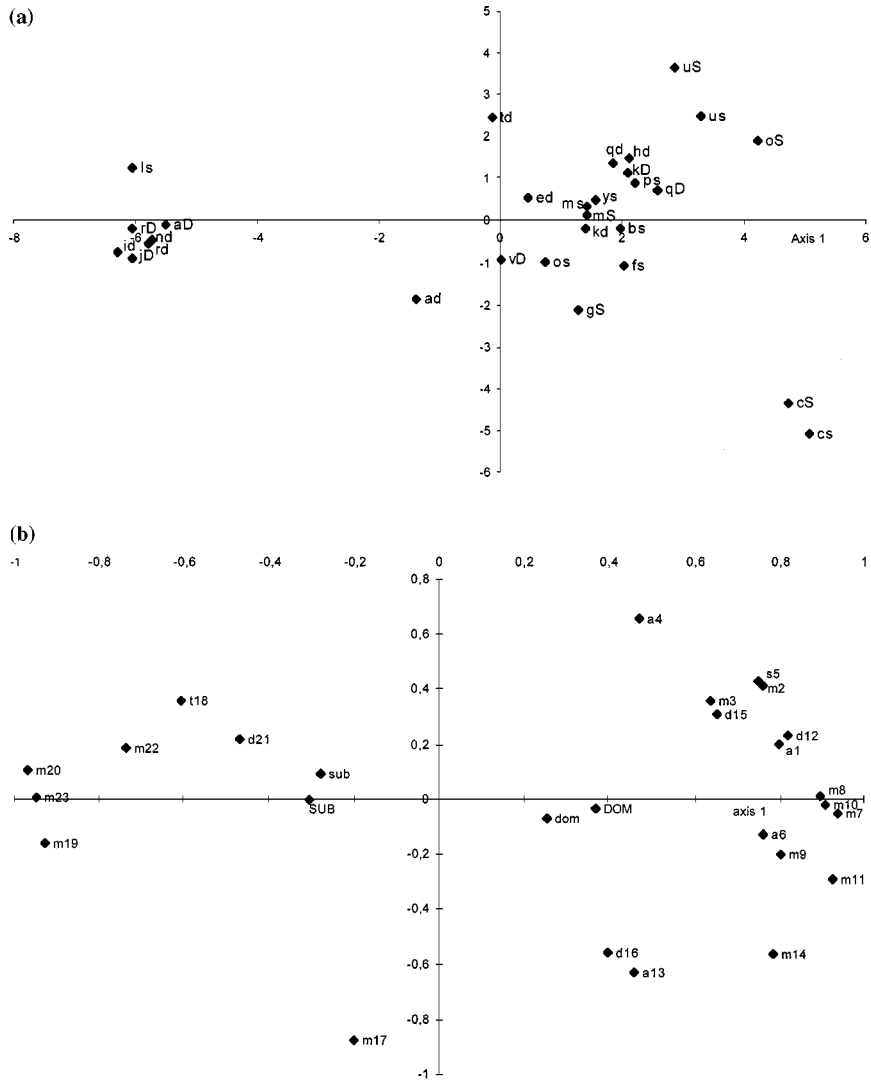


FIG. 5. (a) Principal component analysis of 23 variables (proportions of hydrocarbons) obtained in 29 SPME from 13 individuals sampled once or eight individuals sampled twice (set B). Projection was performed on factorial plane 1–2. Axis 1 accounted for 56.2% of total variance and axis 2 for 14.3%. Samples of three (xD)- or two (xd) -times dominant individuals and three (xS)- or two (xs) -times subordinate individuals were compared. The letter in front of the status designates the individual insect. (b) Projection of the hydrocarbons on axes 1 and 2. Abbreviations as in Figure 3. Mean values for each status were introduced in the PCA as supplementary variables and were encoded as follow: DOM = three-times dominant individuals, dom = two-times dominant individuals, SUB = three-times subordinate individuals, sub = two-times subordinate individuals.

After eliminating the extraction mode effect, we observed a strong correlation between cuticular hydrocarbon profile and individual status (first PCA axis, 40.4% of variance). Dominant individuals were characterized by higher proportions of 11-, 13-MeC_{36}, peak m20; 13-, 15-MeC_{38}, peak m23; and 11-, 13-, 15-MeC_{35}, peak m19; whereas subordinate individuals were characterized by higher proportions of 7-, 9-, 11-MeC_{31}, peak m7; 3-MeC_{31}, peak m9; 3-, 8-MeC_{32}, peak m10; and 9-, 10-, 11-, 12-MeC_{32}, peak m11. These differences could allow individuals meeting for the first time to recognize each other by antennal palpation (Ewing, 1967). In subsequent encounters, individuals manifest dominant or subordinate behavior without making contact, probably by means of volatile substances (Breed et al., 1980).

Two previous studies, i.e., Moore et al. (1995) and Moore (1997), showed that the ratio between two sex pheromone components varied according to individual status. Everaerts et al. (1997) stated that dominant males present a hydrocarbon profile different from that of subordinate males even though separation of these two categories was not clear-cut. These findings and our study suggest that both cuticular hydrocarbon profile and sexual pheromone blend are involved in establishing and maintaining dominance status. This conclusion is further supported by the similar behavior observed during agonistic encounters establishing individual status and mating rituals. In both cases, males expand the abdomen to expose the sternal gland where sexual pheromone is produced (Ewing, 1967; Sreng, 1979).

Laboratory tests show that the outcome of an encounter involving an untried male, regardless of its aggressive potential, depends mainly on the status of the opponent, especially if the latter has already been dominant (Ewing, 1967; Manning and Johnstone, 1970; Breed et al., 1980; Schal and Bell, 1983). Thus physiological changes induced by these agonistic encounters, such as modifications of the chemical signature, could be essential in establishing the dominance status.

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SYNTHESES OF FEMALE SEX PHEROMONE PRECURSORS OF PINE SAWFLY SPECIES AND OF SOME STRUCTURALLY RELATED METHYL-BRANCHED LONG-CHAIN 2-ALKANOLS

ERIK HEDENSTRÖM* and FREDRIK ANDERSSON

Chemistry, Department of Natural and Environmental Sciences
Mid Sweden University
SE-851 70 Sundsvall, Sweden

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Abstract—3,7-Dimethyl-2-undecanol, 3,7,9-trimethyl-2-tridecanol, and 3,7,11-trimethyl-2-tridecanol were synthesized as racemic mixtures in moderate yields. The alcohols are known precursors of the female sex pheromones of the pine sawfly species *Diprion nipponica*, *Macrodiprion nemoralis*, and *Microdiprion pallipes*, respectively. Stereoisomeric mixtures of 3,8,12-trimethyl-2-tridecanol, *erythro*-(2*R*,3*R*,11*R*/*S*)-3,11-dimethyl-2-tetradecanol, 3,5-dimethyl-2-tetradecanol, and 5,7-dimethyl-2-tetradecanol, structurally related to sex pheromone alcohol precursors of pine sawfly species, were also synthesized in moderate yields. The key reaction in the syntheses was the ring opening of γ-butyrolactones by using different alkyl lithiums as nucleophiles.

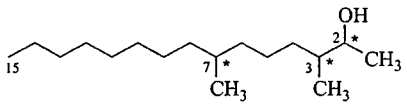
Key Words—Sex pheromone, pine sawfly, synthesis, *Diprion nipponica*, *Macrodiprion nemoralis*, *Microdiprion pallipes.*

INTRODUCTION

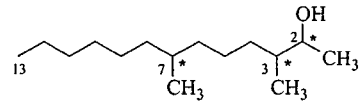
We have long been interested in the sex pheromones of pine sawflies, and our work has thus far included identification, syntheses, and biological activities of such chemical substances (for recent examples see Bergström et al., 1998; Karlsson and Hedenström, 1999; Larsson et al., 2001; Wassgren et al., 2000). In the northern hemisphere, some pine sawfly species are severe pests on pines (Smith, 1993). Consequently, our interest is to monitor and to control pine sawfly populations by using mass trapping or mating disruption techniques (for two recent examples see Rieske

* To whom correspondence should be addressed. E-mail: erik.hedenstrom@mh.se

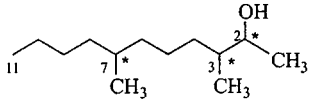
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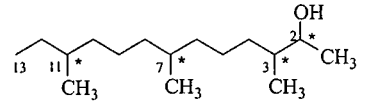
3,7-Dimethyl-2-pentadecanol (**1**)
Neodiprion spp and *Diprion similis*
erythro-2*S*,3*S*,7*S* and *threo*-2*S*,3*R*,7*R*



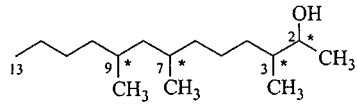
3,7-Dimethyl-2-tridecanol (**2**)
Diprion pini
threo-2*S*,3*R*,7*R*



3,7-Dimethyl-2-undecanol (**3**)
Diprion nipponica
threo-2*S*,3*R*,7*R*/*S*



3,7,11-Trimethyl-2-tridecanol (**4**)
Microdiprion pallipes
erythro-2*S*,3*S*,7*S*,11*S* and *erythro*-2*S*,3*S*,7*S*,11*R*



3,7,9-Trimethyl-2-tridecanol (**5**)
Macrodiprion nemoralis
threo-2*S*,3*R*,7*R*,9*S*

FIG. 1. The sex pheromone precursor alcohols **1**–**5** identified so far in females of pine sawfly species. The syntheses of compounds **3**–**5** as stereoisomeric mixtures are presented in this paper.

et al., 2001; Martini et al., 2002). Several structurally similar sex pheromone precursor alcohols have been identified in females of pine sawfly species (Anderbrant, 1993; Bergström et al., 1995, 1998; Tai et al., 1998; Wassgren et al., 2000; Larsson et al., 2001). As sex pheromone, most of the pine sawfly species employ esters of *erythro*-(2*S*,3*S*,7*S*)- or *threo*-(2*S*,3*R*,7*R*)-3,7-dimethyl-2-pentadecanol (**1**, diprionol, Figure 1). From virgin female extracts of the pine sawfly *Neodiprion sertifer*, only the precursor alcohol **1** has been isolated and no trace of the behavioral active ester was found (Wassgren et al., 1992). So far, little is known about the pheromone biosynthesis in pine sawfly species, and no production site has been found for the pheromone or the precursor alcohol. The alcohol **1** has been found in different parts of the female *N. sertifer* (Wassgren et al., 1992) and probably is stored and then esterified just before emission, as suggested earlier (Jewett et al., 1976).

The sex pheromone precursors identified in female pine sawfly species are basically methyl-branched long-chained 2-alcohols that may be divided into two structural groups. One group is based on different lengths of the carbon chain,

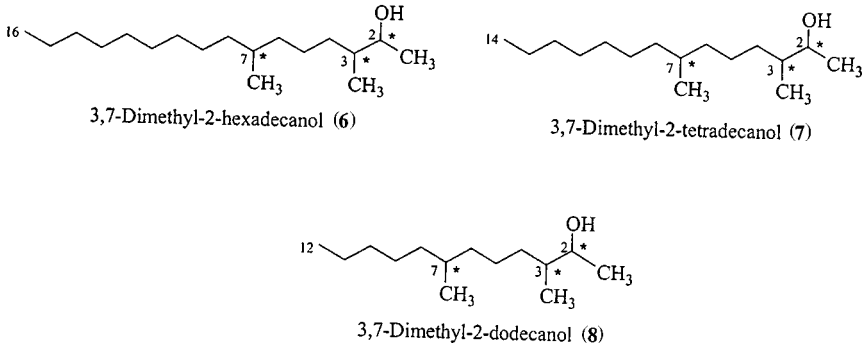


FIG. 2. Lower and higher homologs identified in females of pine sawflies *N. sertifer* (**6** and **7**) and *D. pini* (**6**–**8**) together with the species specific sex pheromone precursor, alcohols **1** and **2**, respectively.

with methyl branching on carbons 3 and 7 (3,7-dimethyl-2-pentadecanol (**1**); 3,7-dimethyl-2-tridecanol (**2**); and 3,7-dimethyl-2-undecanol (**3**); Figure 1). From female extracts of the pine sawflies *N. sertifer* (Hedenström et al., 1992; Wassgren et al., 1992) and *Diprion pini* (Bergström et al., 1995), some minor (1–5%) pheromone components were identified (3,7-dimethyl-2-hexadecanol (**6**); 3,7-dimethyl-2-tetradecanol (**7**); and 3,7-dimethyl-2-dodecanol (**8**); Figure 2). These minor compounds gave a GC-EAD response but showed no activity in field tests performed with either pine sawfly species (Wassgren et al., 1992; Bergström et al., 1995). The other structural group is based on the same 13-carbon chain length but includes yet another methyl branching on a carbon different from carbons 3 and 7, i.e., on carbon 9 or 11 (3,7,11-trimethyl-2-tridecanol, (**4**); and 3,7,9-trimethyl-2-tridecanol (**5**); Figure 1). The propionate of 3,7,11-trimethyl-2-tridecanol (**4**), and the acetate of 3,7,9-trimethyl-2-tridecanol (**5**), have been found to be the sex pheromones of *Microdiprion pallipes* (Bergström et al., 1998; Nakamura and Mori, 1999; Larsson et al., 2001) and *Macrodiprion nemoralis* (Karlsson and Hedenström, 1999; Wassgren et al., 2000), respectively.

To confirm the structure of future pheromone candidates that likely will be found in other pine sawfly species, we need access to an extensive library of structurally related compounds for screening their mass spectra and gas chromatography (GC) retention times and relating the observed variation to the differences in structure. As part of this work, we previously synthesized some structural analogs of diprionol, representing a third group of compounds with one methyl group missing [(2*S*,3*S*)-3-methyl-2-pentadecanol (**9**); (2*S*,7*S*)-7-methyl-2-pentadecanol (**10**); and (2*S*,6*S*)-2,6-dimethyl-1-tetradecanol (**11**); Figure 3] (Hedenström et al., 1992). The biological activity of the acetates of compounds **9**–**11** have been tested on the pine sawfly *N. sertifer* in Sweden (Anderbrant et al.,

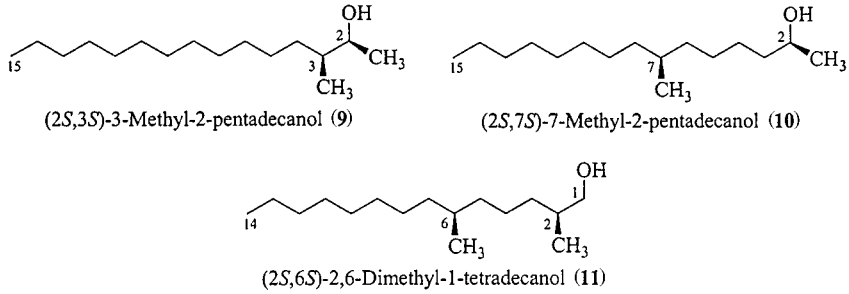


FIG. 3. The acetates of stereoisomeric pure diprionol analogues **9**–**11**. In a field study performed in Sweden on the pine sawfly *N. sertifer*, no biological activity was found.

in personal communication). None of the analogous acetates showed any activity in the field. In field experiments performed in Japan on the same species (Tai et al., 1992), both the *erythro*-(2*S*,3*S*) and the *threo*-(2*S*,3*R*) isomers of analog **9** were active.

We present in this paper the syntheses of some novel substances. One compound, 3,8,12-trimethyl-2-tridecanol (**12**); (Figure 4), which is structurally related to compounds **4** and **5** (Figure 1), and three others represent a new, fourth group of compounds that are structurally related to sex pheromones of pine sawflies. This group has the same 14-carbon chain length but a variation in the attachment of the two methyl groups [*erythro*-(2*R*,3*R*,11*R*/*S*)-3,11-dimethyl-2-tetradecanol (**13**); 3,5-dimethyl-2-tetradecanol (**14**); and 5,7-dimethyl-2-tetradecanol (**15**), Figure 4].

We also present here the syntheses of the 16 stereoisomeric mixtures of 3,7,11- and 3,7,9-trimethyl-2-tridecanol, compounds **4** and **5**, respectively, by somewhat

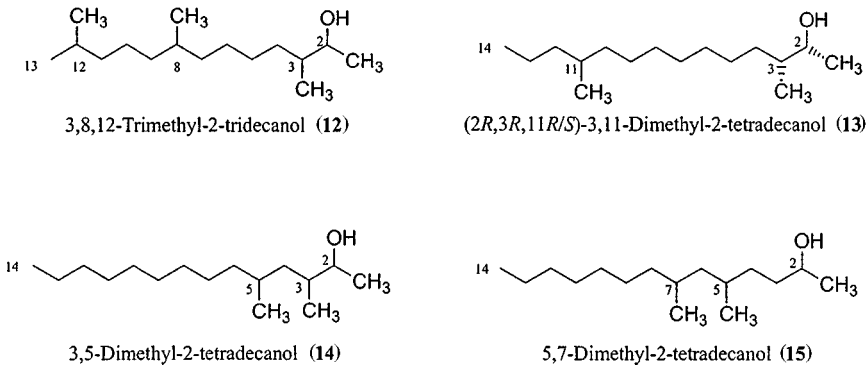


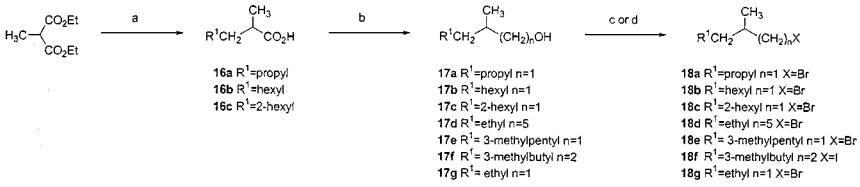
FIG. 4. Stereoisomeric mixtures of methyl-branched, long-chained alcohols **12**–**15** that are structurally related to sex pheromone precursors in pine sawfly species. Their syntheses are presented in this paper.

different procedures from those previously published (Karlsson and Hedenström, 1999; Larsson et al., 2001). The synthesis of 3,7-dimethyl-2-undecanol (**3**) as an eight stereoisomeric mixture is also presented. The propionate of this compound was recently identified as the sex pheromone of *Diprion nipponica* (Tai et al., 1998).

METHODS AND MATERIALS

General Chemical Procedures and Instruments

Unless otherwise stated, commercially available chemicals were used. 2-Methyl-1-pentanol (**17g**), 3,7-dimethyl-1-octanol (**17f**), γ-butyrolactone (**20**), and 4-methyl-γ-butyrolactone (**21**) were purchased from Aldrich and distilled prior to use. 3,4-Dimethyl-γ-butyrolactone (**19**) was prepared according to Hedenström et al. (1992). The *trans–cis* ratio was 65:35 according to GC [iso 115°C, retention time: *cis* (minor): 4.15 min; *trans* (major): 5.34 min]. The analytical and spectral data were identical to those reported in Hedenström et al. (1992). Dry acetone (Na_2CO_3), diethyl ether ($LiAlH_4$), diethyleneglycole (NaOH), pyridine (CaH_2), and THF (K and benzophenone) were distilled from the indicated drying agents. Reactions sensitive to moisture and/or oxygen were carried out under argon. Preparative liquid chromatography was performed on straight-phase silica gel (Merck 60, 230–400 mesh, 0.040–0.063 mm, 10–50 g/g of product mixture) by using a gradient elution technique with an increasing concentration of distilled ethyl acetate in distilled cyclohexane or of distilled diethyl ether in distilled pentane. Thin-layer chromatography (TLC) was performed to monitor the progress of the reactions on silica gel plates (Merck 60, precoated aluminum foil), using ethyl acetate (20 or 40%) in hexane as an eluant, and plates were developed by means of ultraviolet irradiation and/or by spraying with vanillin in sulfuric acid and heating at 120°C. Unless otherwise stated, GC analyses were carried out by using a capillary column [J & W DB-Wax, 30 m $\times$ 0.32 mm ID, $d_f = 0.25\ \mu m$, carrier gas He (103 kPa), split ratio 1:30]. Mass spectral analyses were carried out on a Saturn 2000 GC/MS/MS instrument, coupled to a Varian 3800 GC instrument, using a capillary column [VA-5MS, 30 m $\times$ 0.32 mm ID, $d_f = 0.25\ \mu m$, carrier gas He (60 kPa), split ratio 1:20]. Boiling points are uncorrected and are, unless otherwise stated, given as air bath temperatures (bath temperature/millimeters if mercury) in a bulb-to-bulb apparatus (Büchi GKR-51). IR spectra were recorded as concentrated substance between NaCl or KBr plates with a Perkin Elmer 782 or Perkin Elmer 16PC FT-IR spectrometer. NMR spectra were recorded in $CDCl_3$ with tetramethylsilane as an internal standard using either a Jeol EX270 (270 MHz ^1H and 67.8 MHz ^{13}C) or a Bruker DMX 250 (250 MHz ^1H and 62.9 MHz ^{13}C) instrument. Optical rotation was measured on a Perkin Elmer 241 polarimeter using a 1-dm cell. Elemental analyses were performed by Mikro Kemi AB, Uppsala, Sweden.



SCHEME 1. a) 1) Na(s), EtOH, Δx 2) butylbromide, heptylbromide or 1-bromo-2-methylhexane, Δx 3) KOH, EtOH/H_2O, Δx 4) Δx; b) $LiAlH_4$, Et_2O; c) Br_2, PPh_3, CH_2Cl_2, 0°C 4 hr; d) I_2, PPh_3, imidazole, 0°C, 2 hr.

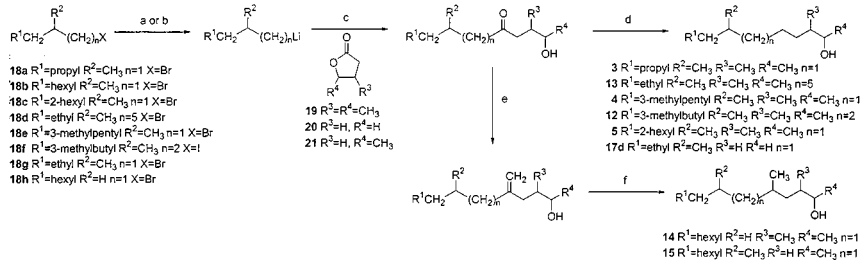
*Synthesis of 3,7-Dimethyl-2-Undecanol (**3**)*

*2-Methylhexanoic Acid (**16a**) (Scheme 1).* The compound was prepared by malonic ester synthesis using the method in Karrer et al. (1930) but from 1-bromobutane. The acid obtained was pure as judged by GC. Bp 120–122°C/14 mm Hg [bp 109–110°C/17 mm Hg for (*S*)-2-methylhexanoic acid (Terashima et al., 1979)]. $n_D^{20} = 1.4249 \pm 0.0002$ [$n_D^{20} = 1.4195$ (Lide, 1999)]. IR data were similar to data for (*S*)-2-methylhexanoic acid (Terashima et al., 1979). The ^{1}H NMR spectral data were identical to those reported in the literature (Lubell et al., 1990) for (*R*)-2-methylhexanoic acid.

*2-Methyl-1-Hexanol (**17a**) (Scheme 1).* This compound was obtained from the above prepared 2-methylhexanoic acid (**16a**) via $LiAlH_4$ reduction (Edlund et al., 1996). It was obtained (16 g, 0.137 mol, 94%) in a >99.5% purity, as judged by GC. Bp 164–165°C/760 mm Hg [bp 112–113.5°C/100 mm Hg (Wender et al., 1950)]. n_D^{20} was identical to that reported in the literature (Mori and Horikiri, 1996) for (*R*)-2-methyl-1-hexanol. IR and ^{1}H NMR spectral data were identical to those reported in the literature (Decicco and Grover, 1996) for (*S*)-2-methyl-1-hexanol.

*1-Bromo-2-Methylhexane (**18a**) (Scheme 1).* This was prepared as described below for 1-bromo-2-methylnonane (**18b**) but from 2-methyl-1-hexanol (**17a**). Distillation {bp 72°C/10.6 mm Hg [bp 90–92°C/40 mm Hg (Manabe et al., 1985)]} gave the pure product as judged by GC (19.3 g, 0.108 mol, 80%). $n_D^{20} = 1.4509 \pm 0.0002$ [$n_D^{25} = 1.4460$ (Manabe et al., 1985)]. IR and ^{1}H NMR data were similar to data in Kurosawa et al. (1997) for (2*S*)-1-bromo-2-methylhexane.

*3,7-Dimethyl-2-Undecanol (**3**) (Scheme 2).* A mixture of stereoisomers was prepared with the preparative procedure used for 3,7-dimethyl-2-dodecanol (Bergström et al., 1995) with slight modification. 3,4-Dimethyl-γ-butyrolactone (**19**), (*trans–cis* 65:35) and 1-bromo-2-methylhexane (**18a**) were used as starting materials, and before adding the bromide to 3.5 equivalents of lithium in Et_2O, the oxide on the surface of the metal was removed with one equivalent of *t*-butanol at 10°C, the Et_2O was removed, and the metal was washed twice with dry Et_2O. Thereafter, we followed the method described in Bergström et al. (1995). The compound was obtained in a 30% yield, and the *threo–erythro* ratio was 65:35 as analyzed

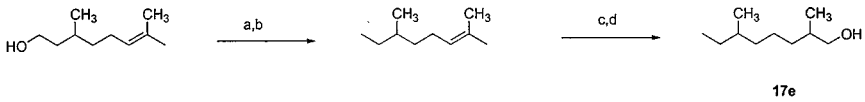


SCHEME 2. a) Li(s), Et_2O, -10°C, 2 hr; b) *t*-Bu-Li, *n*-pentane/Et_2O, -78°C, 1 hr; c) 1) Add to the lactone in Et_2O at -78°C, 4 hr; 2) H_20; d) $(NH_2)_2$, KOH, diethyleneglycol, 180°C, 1 hr, 220°C, 2 hr; e) 1) CH_2PPh_3, benzene, ambient temp., 16 hr; 2) H_2O; f) Pd/C, H_2, MeOH, ambient temp., 72 hr.

by GC [iso 140°C, retention times for the propionate: *threo* (2*S*,3*R* and 2*R*,3*S*, minor): 8.30 min; *erythro* (2*S*,3*S* and 2*R*,3*R*, major): 8.61 min]. Bp 98°C/0.68 mm Hg. $n_D^{20} = 1.4451 \pm 0.0002$. IR (neat): 3355, 2959, 2924, 2858, 1464, 1378, 1094, 1002, 924, 889, 728, 665 cm^{-1}. ^1H NMR and MS data were similar to published data (Tai et al., 1998) for (2*S*,3*R*,7*R*/*S*)-3,7-dimethyl-2-decanol. ^{13}C NMR (62.9 MHz) δ: 14.13, 14.17, 14.49, 14.52, 19.28, 19.33, 19.62, 19.75, 20.27, 20.28, 23.02, 24.63, 24.66, 24.72, 24.75, 29.30, 29.33, 32.67, 32.68, 32.71, 32.77, 32.88, 32.97, 36.66, 36.84, 37.28, 37.37, 39.76, 39.79, 40.01, 40.04, 71.36, 71.45, 71.74, 71.80 ppm. Anal. calcd for $C_{13}H_{28}O$: C 77.9%, H 14.1%; found: C 77.9%, H 14.2%.

*Synthesis of 3,7,11-Trimethyl-2-Tridecanol (**4**)*

*2,6-Dimethyl-1-Octanol (**17e**) (Scheme 3).* The 2,6-dimethyloct-2-ene-1-tossylate was prepared similar to Bergström et al. (1998), but starting from citronellol. The tossylate was obtained in quantitative yield, the purity was checked by IR and ^1H NMR, and it was used without further purification. The tossylate (19.1 g, 61.5 mmol) in dry Et_2O (110 ml) was added dropwise to $LiAlH_4$ (2.60 g, 68.5 mmol) in dry Et_2O (50 ml) at ambient temperature. The reaction was heated to reflux for 3.5 hr (no starting material remained according to TLC), cooled to 0°C, and quenched with water (2.6 ml) and 15% aqueous NaOH (2.6 ml) followed by



SCHEME 3. a) TsCl, pyridine, 5°C, 4 hr; b) 1) $LiAlH_4$, Et_2O, Δx 3.5 hr; 2) H_2O; c) 1) SeO_2, EtOH 2) $NaBH_4$, EtOH, 2 hr; d) RaNi, H_2, MeOH/H_2O, 72 hr.

H_2O (2.6 ml). After refluxing for 1 hr, the precipitate was filtered, and the solution was dried ($MgSO_4$). The Et_2O was distilled off through a column, and the residual oil was chromatographed (eluted with pentane and distilled off through a column) and distilled to give 6.47 g (46.2 mmol, 75%) of the pure alkene (>99% as judged by GC). Bp, n_D^{20}, IR and ^{1}H NMR spectral data were identical to those reported by Mori and Harashima (1993) for (6*R*)-2,6-dimethyl-2-octen. ^{13}C NMR data were similar to those of Naoshima et al. (1988) for (6*S*)-2,6-dimethyl-2-octen]. MS (EI) data were similar to Rossi and Salvadori (1979) for (6*R*)-2,6-dimethyl-2-octen.

The alkene (2.06 g, 14.7 mmol) was added to a solution of SeO_2 (1.23 g, 11.1 mmol) in EtOH (210 ml) and refluxed. A mixture of the allylic alcohol and the α,β-unsaturated aldehyde was formed and after 30 hr, MeOH was added and the selenium residues removed by filtration. Evaporation of the solvent was followed by extraction with Et_2O (3 × 50 ml). The combined organic phases were washed with aqueous saturated Na_2CO_3 and dried ($MgSO_4$). Liquid chromatography and removal of solvent yielded a mixture of the allylic alcohol and the α,β-unsaturated aldehyde (1.17 g, 7.58 mmol, 52%). This was dissolved in absolute EtOH (30 ml) and added drop by drop to a suspension of $NaBH_4$ (0.41 g, 10.7 mmol) in EtOH (80 ml) at 5°C. After 3.5 hr, TLC indicated no aldehyde, and H_2O (115 ml) and brine (50 ml) were added. Extraction with Et_2O (3 × 100 ml) and drying ($MgSO_4$) of the combined extracts gave after liquid chromatography and distillation {bp 70°C/3 mm Hg [bp 100–103°C/10 torr (Webb, 1961)]} 0.99 g (6.33 mmol, 84%) of the unsaturated pure alcohol. $n_D^{20} = 1.4590 \pm 0.0002$ [$n_D^{22} = 1.4541$ for (*E*)-(6*R*)-2,6 dimethyl-2-octen-1-ol (Mori and Harashima, 1993)]. IR data were similar to Mori and Harashima (1993) for (*E*)-(6*R*)-2,6-dimethyl-2-octen-1-ol. ^{1}H NMR and MS (EI) spectral data were identical to those of Ghisalberti et al. (1991) for (6*R*)-2,6-dimethyl-2-octen-1-ol. Compound **17e** was obtained as a mixture of isomers by RaNi reduction of 2,6-dimethyl-2-octen-1-ol according to Högberg et al. (1992). Bp 90°C/6.8 mm Hg [bp 60°C/1 mm Hg for (2*R*,6*R*)-2,6-dimethyl-1-octanol (Cheskis et al., 1988)]. $n_D^{20} = 1.4249 \pm 0.0002$ [$n_D^{20} = 1.4371$ for (2*R*,6*R*)-2,6-Dimethyl-1-octanol (Cheskis et al., 1988)]. The IR and ^{1}H NMR spectral data were identical to those reported by Chênevert and Desjardins (1996) for (2*R*,6*R*)-2,6-dimethyl-1-octanol.

*1-Bromo-2,6-Dimethyloctane (**18e**) (Scheme 1).* This was prepared as below for 1-bromo-2-methylnonane (**18b**) but from 2,6-dimethyl-1-octanol (**17e**). Distillation {bp 70–72°C/8 mm Hg [bp 45°C/0.7 mm Hg for (2*R*,6*R*)-1-bromo-2,6-dimethyloctane (Cheskis et al.,1988)]} gave the pure product, as judged by GC. $n_D^{20} = 1.4530 \pm 0.0002$ [$n_D^{20} = 1.4565$ for (2*R*,6*R*)-1-bromo-2,6-dimethyloctane (Cheskis et al., 1988)]. The IR, ^{1}H NMR, and MS (EI) spectral data were identical to those reported by Cheskis et al. (1988) for (2*R*,6*R*)-1-bromo-2,6-dimethyloctane.

*3,7,11-Trimethyl-2-Tridecanol (**4**) (Scheme 2).* This was prepared as above for 3,7-dimethyl-2-undecanol (**3**) but starting from 3,4-dimethyl-γ-butyrolactone (**19**), (*trans–cis* 65:35) and 1-bromo-2,6-dimethyloctane (**18e**). It showed by GC

a *threo–erythro* ratio of 62:38 [iso 120°C, retention times: *erythro* (2*S*,3*S*,7*R*/*S*, 11*R*/*S* and 2*R*,3*R*,7*R*/*S*,11*R*/*S*, minor): 31.33 min; *threo* (2*S*,3*R*,7*R*/*S*,11*R*/*S* and 2*R*,3*S*,7*R*/*S*,11*R*/*S*, major): 31.83 min]. Bp 95°C/0.65 mm Hg. $n_D^{20} = 1.4508 \pm 0.0002$. IR (neat): 3358, 2961, 2926, 2872, 1462, 1377, 1099, 1003, 925, 888, 666 cm^{-1}. ^1H NMR and ^{13}C NMR spectral data were identical to those reported by Larsson et al. (2001). MS (EI) *m*/*z* (relative intensity): 227 (5%), 224 (M^+-H_2O, 3), 196 (3), 168 (2), 140 (9), 125 (87), 111 (46), 97 (55), 85 (70), 83 (55), 71 (90), 69 (80), 57 (100), 55 (73), 45 (84), 43 (55), 41 (72).

*Synthesis of 3,7,9-Trimethyl-2-Tridecanol (**5**)*

*2,4-Dimethyloctanoic Acid (**16c**) (Scheme 1).* This compound was prepared as 2-methylhexanoic acid (**16a**) above but from 1-bromo-2-methylhexane (**18a**). We obtained 9.1 g (0.053 mol, 57%) of the acid as a racemic mixture of *syn–anti* (45:55) products. Bp 127–130°C/9 mm Hg. $n_D^{20} = 1.4300 \pm 0.0002$. IR, ^1H NMR, and ^{13}C NMR spectral data were identical to those reported by Decicco and Grover (1996) for (2*R*,4*S*)- and (2*S*,4*S*)-2,4-dimethyloctanoic acid).

*2,4-Dimethyl-1-Octanol (**17c**) (Scheme 1).* This compound was obtained from the acid **16c** *via* $LiAlH_4$ reduction as for **17a**. The alcohol (3.1 g, 0.053 mol, 86%) was obtained as a racemic mixture of *syn–anti* (45:55) products and in >99.2% purity as judged by GC. Bp 130°C/9.9 mm Hg [bp 90°C/3 mbar for (2*R*,4*S*)-2,4-dimethyl-1-octanol (Karlsson and Hedenström, 1999)]. $n_D^{20} = 1.4332 \pm 0.0002$. IR (neat): 3342, 2958, 2925, 2879, 1463, 1379, 1038 cm^{-1}. ^1H NMR and ^{13}C NMR were similar to those reported by Karlsson and Hedenström (1999) for (2*R*,4*S*)- and (2*S*,4*S*)-2,4-dimethyl-1-octanol).

*1-Bromo-2,4-Dimethyloctane (**18c**) (Scheme 1).* This was prepared as **18b** but from 2,4-dimethyl-1-octanol (**17c**). Distillation {bp 110°C/6 mm Hg [bp 90°C/7 mbar for (2*S*,4*S*)-1-bromo-2,4-dimethyloctane (Karlsson and Högberg, 2000)]} gave the pure product as judged by GC. $n_D^{20} = 1.4569 \pm 0.0002$. IR (neat): 2958, 2927, 2871, 1459, 1379, 1228, 665, 654 cm^{-1}. ^1H NMR data were similar to those of Karlsson and Högberg (2000) for (2*S*,4*S*)-1-bromo-2,4-dimethyloctane. Anal. calcd for $C_{10}H_{21}Br$: C 54.3%, H 9.6%; found: C 53.9%, H 9.5%.

*3,7,9-Trimethyl-2-Tridecanol (**5**) (Scheme 2).* This was prepared as above for 3,7-dimethyl-2-undecanol (**3**) but starting from 3,4-dimethyl-γ-butyrolactone (**19**), (*trans–cis* 65:35) and 1-bromo-2,4-dimethyloctane (**18c**). It showed a *threo–erythro* ratio of 64:36 analyzed by GC [CP-Wax 52CB, 30 m × 0.25 mm ID, $d_f = 0.25$ μm, carrier gas He (110 kPa), split ratio 1:30, 80°C, 0 min, then programmed 1.0°C/min up to 250°C, retention times: *erythro* (2*S*,3*S*,7*R*/*S*, 9*R*/*S* and/or 2*R*,3*R*,7*R*/*S*,9*R*/*S*, minor): 57.63 min; *erythro* (2*S*,3*S*,7*R*/*S*,9*R*/*S* and/or 2*R*,3*R*,7*R*/*S*,9*R*/*S*) and *threo* (2*S*,3*R*,7*R*/*S*,9*R*/*S* and/or 2*R*,3*S*,7*R*/*S*, 9*R*/*S*): 58.86 min; *threo* (2*S*,3*R*,7*R*/*S*,9*R*/*S* and/or 2*R*,3*S*,7*R*/*S*,9*R*/*S*, major): 59.97 min]. Bp, ^1H NMR and ^{13}C NMR spectral data were identical to those

reported by Karlsson and Hedenström (1999). $n_D^{20} = 1.4510 \pm 0.0002$. IR (neat): 3354, 2957, 2926, 2872, 1461, 1378, 1097, 1066, 1002, 923, 728 cm^{-1}. MS (EI) *m*/*z* (relative intensity): 227 (1%), 166 (2), 154 (2), 139 (7), 125 (40), 111 (40), 97 (39), 85 (58), 83 (49), 71 (67), 69 (72), 57 (100), 55 (59), 45 (64), 43 (53), 41 (74).

*Synthesis of 3,8,12-Trimethyl-2-Tridecanol (**12**)*

*1-Iodo-3,7-Dimethyloctane (**18f**) (Scheme 1).* Preparation of this compound was according to Marshall and De Hoff (1987) but from 3,7-dimethyl-1-octanol (**17f**). Distillation gave the pure product as judged by GC. Bp and n_D^{20} were identical to those reported in Schlenk (1973). IR and ^{1}H NMR data were similar to data reported in Nagai (1976).

*3,8,12-Trimethyl-2-Tridecanol (**12**) (Scheme 2).* This was prepared as for 3,7-dimethyl-2-undecanol (**3**) above but starting from 3,4-dimethyl-γ-butyrolactone (**19**) (*trans–cis* 65:35) and 1-iodo-3,7-dimethyloctane (**18f**). It showed a *threo–erythro* ratio of 65:35 by GC [iso 120°C, retention times: *erythro* (2*S*,3*S*,8*R*/*S* and 2*R*,3*R*,8*R*/*S*, minor): 28.20 min; *threo* (2*S*,3*R*,8*R*/*S* and 2*R*,3*S*,8*R*/*S*, major): 28.99 min]. Bp 120°C/0.48 mm Hg. $n_D^{20} = 1.4486 \pm 0.0002$. IR (neat): 3355, 2955, 2926, 2869, 1466, 1377, 1099, 1004, 924, 665 cm^{-1}. ^{1}H NMR (250 MHz) δ: 0.83–0.90 (6H, m), 0.87 (6H, d, $J = 6.5$ Hz), 1.01–1.60 (18H, m), 1.13 (1.95H, d, $J = 6.3$ Hz), 1.15 (1.05H, d, $J = 6.9$ Hz), 3.62–3.75 (1H, m) ppm. ^{13}C NMR (62.9 MHz) δ: 14.20, 14.55, 19.35, 19.70, 19.74, 20.31, 22.64, 22.73, 24.81, 27.40, 27.43, 27.62, 27.64, 27.74, 27.99, 32.59, 32.62, 32.71, 32.78, 32.80, 37.09, 37.12, 37.29, 37.35, 39.37, 39.83, 40.06, 71.44, 71.46, 71.80, 71.82 ppm. MS (EI) *m*/*z* (relative intensity): 227 (5%), 224 (M^+-H_2O, 3), 196 (2), 154 (4), 139 (12), 138 (12), 126 (17), 125 (18), 111 (74), 97 (27), 85 (38), 83 (47), 71 (84), 69 (54), 57 (84), 55 (55), 45 (100), 43 (62), 41 (75). Anal. calcd for $C_{16}H_{34}O$: C 79.3%, H 14.1%; found: C 79.1%, H 14.5%.

Synthesis of Erythro-*(2*R,*3*R,*11*R/S*)-3,11-Dimethyl-2-Tetradecanol (**13**)*

*1-Bromo-2-Methylpentane (**18g**) (Scheme 1).* This was prepared as below for 1-bromo-2-methylnonane (**18b**) but from 2-methyl-1-pentanol (**17g**) and gave the pure product after flash chromatography and distillation, as judged by GC. Bp 55°C/60 mm Hg [bp 128–130°C/760 mm Hg (Ingold et al., 1986)]. $n_D^{20} = 1.4471 \pm 0.0002$ [$n_D^{20} = 1.4495$ (Lide, 1999)]. IR data were similar to data reported in Crowder and Jalilian (1978)]. ^{1}H NMR were similar to data in Ingold et al. (1986).

*6-Methyl-1-Nonanol (**17d**) (Scheme 1).* This was prepared from 1-bromo-2-methylpentane (**18g**) and γ-butyrolactone (**20**) using the procedure as for 3,7-dimethyl-2-dodecanol (Bergström et al., 1995). The compound was >97% pure as judged by GC. Bp 79–82°C/11 mm Hg [bp 110–111°C/10 mm Hg (Weitzel

and Wojhan, 1951)]. $n_D^{20} = 1.4123 \pm 0.0002$. IR and ^{1}H NMR data were similar to data in Génard and Patin (1991).

*1-Bromo-6-Methylnonane (**18d**) (Scheme 1).* This was prepared as for 1-bromo-2-methylnonane (**18b**) but from 6-methyl-1-nonanol (**17d**). The product was checked by ^{1}H NMR and then used in the next step without further purification or characterization.

Erythro-*(2*R,*3*R,*11*R/S)-3,11-*Dimethyl-2-Tetradecanol (**13**) (Scheme 2).* The two-diastereomeric mixture was prepared using the same preparative procedure as for 3,7-dimethyl-2-dodecanol (Bergström et al., 1995) starting from pure (3*R*,4*R*)-3,4-dimethyl-γ-butyrolactone (**19**) and 1-bromo-6-methylnonane (**18d**). The two-diastereomeric mixture was chemically pure. Bp 130°C/2.5 mm Hg. $n_D^{20} = 1.4502 \pm 0.0002$. $[\alpha]_D^{20} = +11.7$ (*c* 4.14 *n*-hexane). IR (neat): 3356, 2959, 2926, 2855, 1466, 1378, 1096, 1004, 928, 722 cm^{-1}. ^{1}H NMR (250 MHz) δ: 0.84 (3H, d, $J = 6.4$ Hz), 0.88 (3H, t, $J = 6.7$ Hz), 0.88 (3H, d, $J = 6.8$ Hz), 1.00–1.50 (21H, broad), 1.15 (3H, d, $J = 6.4$ Hz), 3.71 (1H, dq, $J = 6.3$, $J = 4.2$ Hz) ppm. ^{13}C NMR (62.9 MHz) δ: 14.16, 14.43, 19.67, 20.15, 20.29, 27.08, 27.37, 29.72, 29.99, 30.02, 32.48, 32.66, 37.09, 39.42, 39.78, 71.44 ppm. MS (EI) *m*/*z* (relative intensity): 227 (5%), 224 (M^+-H_2O, 3), 194 (4), 180 (10), 169 (2), 168 (4), 152 (16), 139 (7), 125 (32), 111 (50), 97 (81), 85 (60), 83 (47), 71 (61), 69 (56), 57 (81), 55 (79), 45 (100), 43 (71), 41 (78). Anal. calcd for $C_{16}H_{34}O$: C 79.3%, H 14.1%; found: C 78.9%, H 13.8%.

*Synthesis of 3,5-Dimethyl-2-Tetradecanol (**14**)*

*3,5-Dimethyl-2-Tetradecanol (**14**) (Scheme 2).* This alcohol was prepared in a slightly modified manner from the below 5,7-dimethyl-2-tetradecanol **15** and started from 3,4-methyl-γ-butyrolactone (**19**), (*trans–cis* 65:35) and 1-bromononane (**18h**). To improve the yield of the Wittig reaction by preventing cyclization of the ketoalcohol to the hemiketal, the isolated ketoalcohol was acetylated quantitatively according to the method of Byström et al. (1981) before performing the Wittig reaction. 3-Methyl-5-methylene-2-tetradecanol so obtained as a mixture of isomers was >95% chemically pure. IR (neat): 3374, 3072, 2959, 2926, 2855, 1718, 1643, 1458, 1378, 1090, 722 cm^{-1}. ^{1}H NMR (270 MHz) δ: 0.85 (3H, d, $J = 6.9$ Hz), 0.88 (3H, t, $J = 6.7$ Hz), 1.17 (3H, d, $J = 6.3$ Hz), 1.20–1.60 (14H, m), 1.60–1.90 (2H, m), 1.80 (1H, bs disappears when shaken with D_2O), 1.90–2.10 (2H, m), 2.12–2.30 (1H, m), 3.60–3.80 (1H, m), 4.75 (2H, d, $J = 9.9$ Hz) ppm. The compound was obtained as a mixture of stereoisomers after hydrogenation. The diastereomeric ratio as propionates was 32:19:17:32 [DB-5, 30 m × 0.32 mm ID, $d_f = 0.25$ μm, carrier gas N_2 (103 kPa), split ratio 1:30, 170°C, 1 min, then programmed 0.5°C/min up to 200°C, retention times for the propionate isomers: 17.7, 18.4, 18.9, and 19.2 min]. Bp 100°C/0.06 mm Hg. $n_D^{20} = 1.4498 \pm 0.0002$. IR (neat): 3350, 2956, 2920, 2869, 2849, 1464, 1376, 1101, 1077, 602 cm^{-1}. ^{1}H NMR (250 MHz) δ:

0.81–0.91 (9H, m), 1.11 (0.48H, d, $J = 4.7$ Hz), 1.13 (0.72H, d, $J = 4.7$ Hz), 1.15 (1.80H, d, $J = 6.4$ Hz), 1.19–1.38 (20H, broad), 1.41 (1H, bs), 3.59–3.75 (1H, m) ppm. ^{13}C NMR (62.9 MHz) δ: 13.94, 14.14, 14.35, 14.42, 18.79, 19.23, 19.35, 20.29, 20.47, 20.69, 22.70, 26.82, 26.85, 27.11, 29.37, 29.66, 29.73, 29.99, 30.02, 30.05, 30.08, 31.93, 36.14, 36.27, 36.90, 37.12, 37.32, 38.25, 39.77, 40.06, 40.51, 40.62, 71.12, 71.69, 72.10, 72.36 ppm. MS (EI) *m*/*z* (relative intensity): 227 (1%), 224 (M^+-H_2O, 3), 195 (9), 169 (2), 168 (6), 153 (19), 152 (19), 141 (6), 140 (8), 139 (6), 125 (18), 111 (38), 97 (87), 85 (50), 83 (37), 71 (82), 69 (60), 57 (100), 55 (68), 45 (53), 43 (66), 41 (65). Anal. calcd for $C_{16}H_{34}O$: C 79.3%, H 14.1%; found: C 79.5%, H 14.0%.

*Synthesis of 5,7-Dimethyl-2-Tetradecanol (**15**)*

*2-Methylnonanoic Acid (**16b**) (Scheme 1).* This was prepared as for **16a** but starting from 1-bromoheptane. The acid was obtained in a 51% yield and >99.5% pure as judged by GC. Bp 76–77°C/0.5 mm Hg [bp 88–90°C/0.5 mm Hg (Nikishin et al., 1974)]. $n_D^{20} = 1.4249 \pm 0.0002$ [$n_D^{25} = 1.4312$ for (*R*)-2-methylnonanoic acid (Levene and Marker, 1932)]. IR data and ^{1}H NMR data were similar to Nikishin et al. (1974).

*2-Methyl-1-Nonanol (**17b**) (Scheme 1).* The compound was obtained from the acid **16b** via $LiAlH_4$ reduction, as above for **17a**, in 92% yield and was pure as judged by GC. Bp 56–57°C/1.5 mm Hg [bp 90°C/22 mm Hg for (*S*)-2-methyl-1-nonanol (Högberg et al., 1992)]. $n_D^{20} = 1.4123 \pm 0.0002$ [$n_D^{20} = 1.4381$ for (*S*)-2-methyl-1-nonanol (Högberg et al., 1992)]. IR and ^{1}H NMR data were similar to Högberg et al. (1992) for (*S*)-2-methyl-1-nonanol.

*1-Bromo-2-Methylnonane (**18b**) (Scheme 1).* The compound was prepared from 2-methyl-1-nonanol (**17b**) (8.06 g, 0.051 mol), which was added dropwise to a solution of triphenylphosphine (17.1 g, 0.111 mol) and bromine (3.4 ml, 0.067 mol) in CH_2Cl_2 at 0°C. The mixture was stirred overnight at ambient temperature. The solvent was evaporated, the residue was triturated with pentane, and the triphenylphosphine oxide was removed by filtration. Evaporation of the solvent, flash chromatography of the remaining oil and distillation {bp 76°C/8 mm Hg [bp 70°C/3 mm Hg (Hedenström et al., 1992)]} gave the pure product (9.91 g, 0.045 mol, 88%). $n_D^{20} = 1.4530 \pm 0.0002$. IR (neat): 2955, 2923, 2868, 2852, 1458, 1377, 653, 622, 602, 592 cm^{-1}. ^{1}H NMR (250 MHz) δ: 0.88 (3H, t, $J = 6.6$ Hz), 1.00 (3H, d, $J = 6.6$ Hz), 1.18–1.49 (12H, broad), 1.72–1.84 (1H, m) 3.32 (1H, dd, $J = 9.8$, $J = 6.2$ Hz), 3.40 (1H, dd, $J = 9.8$, $J = 5.0$ Hz) ppm.

*5,7-Dimethyl-2-Tetradecanol (**15**) (Scheme 2).* This stereoisomeric mixture was prepared using the procedure for 3,7-dimethyl-2-dodecanol (Bergström et al., 1995) with a slight modification. An amount of 4.56 g (20.6 mmol) of 1-bromo-2-methylnonane (**18b**) was added dropwise to 50 mmol finely cut lithium in

20 ml Et_2O at $-5^\circ C$. After stirring for 2 hr, the alkyl lithium was cooled to $-100^\circ C$ and added dropwise to a stirred solution of 1.73 g (17.3 mmol) of 4-methyl-γ-butyrolactone (**21**) in 40 ml of Et_2O at $-100^\circ C$. From the recovered unreacted lithium metal, the alkyl lithium produced was calculated to be 14 mmol (68%). After checking the progress of the reaction by TLC, the reaction was quenched after 5 hr at $-100^\circ C$ with water (44 mmol) in THF, and the mixture was allowed to reach ambient temperature overnight. Thereafter, 150 ml of water and 150 ml of Et_2O were added, and the organic phase was separated. The water phase was extracted with Et_2O (3×150 ml), and the pooled organic phases were washed with brine (100 ml) and dried ($MgSO_4$). Evaporation of the solvent left a yellow oil, which was purified by flash chromatography to give 1.99 g (8.2 mmol) of the ketoalcohol. The identity of the product was checked by IR and ^1H 60 MHz NMR, and the ketoalcohol was used in the next step without further purification or characterization. To the ketoalcohol in 50 ml of dry benzene at ambient temperature was added a dark red solution of an ylide [prepared by refluxing 14.32 g (40 mmol) of flame-dried methyltriphenylphosphoniumbromide in dry benzene (35 ml) and 31 ml of BuLi (1.3 M in *n*-hexane)]. The reaction was stirred overnight and then poured onto ice and water (400 ml). The organic phase was separated, the water phase was extracted with Et_2O (3×150 ml), the combined organic phases were washed with aqueous 0.5 M HCl (100 ml) and aqueous saturated $NaHCO_3$ (100 ml), and dried ($MgSO_4$). The residue gave, after solvent evaporation and flash chromatography, 0.90 g (3.8 mmol) of pure 7-methyl-5-methylene-2-tetradecanol >95% as judged by GC. IR (neat): 3337, 3070, 2956, 2923, 2851, 1643, 1457, 1375, 889, 602 cm^{-1}. ^1H NMR (270 MHz) δ: 0.83 (3H, d, $J = 6.6$ Hz), 0.88 (3H, t, $J = 6.7$ Hz), 1.21 (3H, d, $J = 5.9$ Hz), 1.20–1.40 (14H, m), 1.50–1.70 (2H, m), 1.75 (1H, bs disappears when shaken with D_2O), 1.75–1.83 (1H, m), 2.00–2.18 (2H, m), 3.73–3.87 (1H, m), 4.74 (2H, d, $J = 18.8$ Hz) ppm. 0.83 g (3.47 mmol).

An amount of 0.83 g (3.47 mmol) of this unsaturated alcohol was dissolved in dry methanol, and 0.07 g of 10% Pd/C was added. The reaction mixture was hydrogenated at 1 atm for 72 hr. After removal of the catalyst and solvent, the remaining oil was purified by flash chromatography and distillation to give 0.68 g (2.80 mmol, total yield 16% based on the 4-methyl-γ-butyrolactone (**4**) as a mixture of stereoisomers. The diastereomeric ratio judged by GC as propionates was 23:25:27:25 [iso 140°C, retention times for the propionate isomers: 13.9, 14.4, 15.6, and 15.9 min]. Bp 100°C/0.6 mm Hg. $n_D^{20} = 1.4502 \pm 0.0002$. IR (neat): 3335, 2955, 2921, 2867, 2849, 1462, 1375, 1118, 1069, 602 cm^{-1}. ^1H NMR (250 MHz) δ: 0.80–1.58 (30H, m), 1.19 (3H, d, $J = 6.2$ Hz), 3.70–3.83 (1H, m) ppm. ^{13}C NMR (62.9 MHz) δ: 14.14, 19.45, 19.49, 19.51, 20.22, 20.27, 22.70, 23.47, 23.52, 23.57, 26.92, 26.94, 27.07, 29.41, 29.97, 29.99, 30.02, 30.04, 30.06, 30.10, 30.16, 30.18, 31.94, 32.73, 32.80, 33.87, 33.91, 36.66, 36.70, 36.83, 36.86, 37.98, 44.75, 44.78, 45.06, 45.10, 68.59, 68.63, 68.68 ppm. MS (EI) *m*/*z* (relative intensity): 227 (2%), 181 (1), 167 (29), 140 (6), 139 (3), 125 (88), 111 (29), 97

(64), 85 (38), 83 (83), 71 (45), 69 (100), 57 (96), 55 (88), 45 (54), 43 (59), 41 (78). Anal. calcd for $C_{16}H_{34}O$: C 79.3%, H 14.1%; found: C 79.6%, H 14.1%.

RESULTS AND DISCUSSION

Employing essentially the same synthetic methodology that we used previously, the mixture of stereoisomers of compound **3** was prepared, as shown in Schemes 1 and 2. Racemic 2-methylhexanoic acid (**16a**) was prepared using the malonic ester synthesis sequence according to the method described (Karrer et al., 1930), and after subsequent lithium aluminum hydride ($LiAlH_4$) reduction, 2-methyl-1-hexanol (**17a**) was obtained (Scheme 1). The alcohol was converted by way of the bromide **18a** into the alkyl lithium, which was reacted with a racemic mixture of diastereomers of 3,4-dimethyl-γ-butyrolactone (**19**) to give a ketoalcohol. When exposed to Huang-Minlon reduction, this gave the desired alcohol 3,7-dimethyl-2-undecanol (**3**) as an eight stereoisomer mixture with a *threo–erythro* ratio of 65:35 (Scheme 2).

In the same way, the mixtures of the 16 stereoisomers of 3,7,11-trimethyl-2-tridecanol (**4**) (*threo–erythro*, 65:35), of 3,8,12-trimethyl-2-tridecanol (**12**) (*threo–erythro*, 65:35), and of 3,7,9-trimethyl-2-tridecanol (**5**) (*threo–erythro*, 64:36) were prepared from a racemic mixture of diastereomers of 3,4-dimethyl-γ-butyrolactone (**19**) and alkyl lithiums obtained from 2,6-dimethyl-1-octanol (**17e**) 3,7-dimethyl-1-octanol (**17f**), and 2,4-dimethyl-1-octanol (**17c**), respectively. 2,6-Dimethyl-1-octanol (**17e**) was prepared from citronellol via tossylation, $LiAlH_4$ reduction, allylic oxidation of the obtained 2,6-dimethyl-2-octene with SeO_2, followed by $NaBH_4$ reduction and hydrogenation (RaNi, H_2) to give the desired alcohol (Scheme 3). 2,4-Dimethyl-1-octanol (**17c**) was prepared via the malonic ester synthesis as above but from 1-bromo-2-methylhexane (**18a**), and after subsequent $LiAlH_4$ reduction of the acid **16c**, the alcohol **17c** was obtained (Scheme 1).

To prepare *erythro*-3,11-dimethyl-2-tetradecanol (**13**) as a mixture of two of the possible diastereomers, the following steps were used: racemic 2-methyl-1-pentanol (**17g**) was converted via the bromide **18e** into the alkyl lithium, which was reacted with γ-butyrolactone (**20**) to give a ketoalcohol, after Huang-Minlon reduction, gave the racemic 6-methyl-1-nonanol (**17d**) (Scheme 2). The alkyl lithium, obtained as above but through the alcohol **17d** and the bromide **18d**, was reacted with the pure enantiomer of *cis*-(3*R*,4*R*)-3,4-dimethyl-γ-butyrolactone (**19**) and the ketoalcohol obtained was reduced as above to give compound **13** as two *erythro*-diastereoisomers without any detectable amount of *threo*-diastereomers (Scheme 2).

The alcohols 3,5-dimethyl-2-tetradecanol (**14**) and 5,7-dimethyl-2-tetradecanol (**15**) were prepared as stereoisomeric mixtures from 1-nonyl lithium and 3,4-dimethyl-γ-butyrolactone (**19**) and from 2-methylnonyl lithium and 4-methyl-γ-butyrolactone (**21**), respectively, as above (Scheme 2). Both 1-nonyl

lithium and 2-methylnonyl lithium were prepared for the bromide **18a** but via 1-bromononane (**18h**) or the acid **16b**, the alcohol **17b**, and the bromide **18b**, respectively (Scheme 1). The resulting ketoalcohols were reacted with two equivalents of phosphorus ylide, and the obtained methylene group was hydrogenated to give the products **14** and **15**, respectively (Scheme 2). The alcohols **14** and **15** were converted to the corresponding propionates and analyzed by GC for their diastereomeric ratios, which were found to be 32:19:17:32 and 23:25:27:25, respectively.

In conclusion, we have presented the syntheses of an eight-stereoiomeric mixture of 3,7-dimethyl-2-undecanol (**3**), the precursor of the sex pheromone of *Diprion nipponica* (Tai et al., 1998) and the syntheses of the 16 stereoisomeric mixtures of 3,7,11- and 3,7,9-trimethyl-2-tridecanol (compounds **4** and **5**), the precursors of the sex pheromones of *M. pallipes* (Bergström et al., 1998; Nakamura and Mori, 1999; Larsson et al., 2001) and *M. nemoralis* (Karlsson and Hedenström, 1999; Wassgren et al., 2000), respectively. The propionate and acetate esters of these mixtures of stereoisomers (compounds **4** and **5**) have been tested in field studies and shown to efficiently catch males of *M. pallipes* (Bergström et al., 1998; Nakamura and Mori, 1999) and of *M. nemoralis* (Wassgren et al., 2000). Whether the propionate ester of the eight stereoiomeric mixture of 3,7-dimethyl-2-undecanol (**3**) is capable of catching males of the species *Diprion nipponica* remains to be investigated in field tests. We also have presented the syntheses of some new substances that are structurally related to sex pheromones of pine sawfly species and the biological activities of these substances have not yet been established.

It is important to have access to fast and trustworthy analysis methods for elucidation of pheromone candidates found in different species of insects such as, pine sawflies. Access to libraries of several structurally similar compounds is, therefore, essential for the screening of, for instance, their mass spectra and GC retention times so that structural differences may be recorded and compared with those obtained from the natural source. This work will contribute to such a library, which can be used when identifying new compounds found in pine sawfly species in the future.

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DEFINING A SYNTHETIC PHEROMONE BLEND ATTRACTIVE TO MALE *Heliothis subflexa* UNDER WIND TUNNEL CONDITIONS

NEIL J. VICKERS*

Department of Biology
University of Utah
Salt Lake City, Utah 84112

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Abstract—*Heliothis subflexa* males were flown in a wind tunnel to a variety of combinations of synthetic pheromone components admixed on a filter paper disk. Blends containing (*Z*)-11-hexadecenal (*Z*11–16:Ald, 1000 ng), (*Z*)-9-hexadecenal (*Z*9–16:Ald, 500 ng) and (*Z*)-11-hexadecenol (*Z*11–16:OH, 10–500 ng) elicited upwind flight and source contact in 52–69% of males. All these compounds have previously been isolated and identified from female *H. subflexa* gland extracts and volatile pheromone emissions. Males were not attracted by blends in which *Z*9–16:Ald was omitted (0% source contact). Similarly, blends lacking *Z*11–16:OH were unattractive to male *H. subflexa* (3% or less source contact). Males were extremely sensitive to the presence of *Z*11–16:OH; however, responding in high numbers (57–69% source contact) to blends containing a dosage of 1% (10 ng) or greater *Z*11–16:OH. Males were unresponsive to blends in which *Z*9–16:Ald was replaced with a variety of dosages of (*Z*)-9-tetradecenal, a secondary component of a closely-related congeneric species, *Heliothis virescens*. Another compound present in the blend emitted by conspecific females, (*Z*)-11-hexadecenyl acetate (*Z*11–16:Ac), did not inhibit *H. subflexa* males (69% source contact) when added to the three-component mixture (1:0.5:0.1) at a ratio of 0.1 (100 ng) with respect to *Z*11–16:Ald. These results indicate that *Z*9–16:Ald and *Z*11–16:OH are required in addition to *Z*11–16:Ald to elicit significant levels of upwind flight in *H. subflexa* males. The effects of *Z*11–16:Ac are more subtle, but at the dosage tested in these experiments, this compound does not have an antagonistic effect on upwind flight and source location by *H. subflexa* males.

Key Words—Moths, Heliothine, Lepidoptera, Noctuidae, sex pheromone, behavior.

* E-mail: vickers@biology.utah.edu

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INTRODUCTION

The pheromone communication system of *Heliothis subflexa* (Guenée) (Lepidoptera: Noctuidae) has been studied largely because of the close relationship of this innocuous species to its agriculturally important sister species, *Heliothis virescens*. The ability of these two species to interbreed is an interesting biological phenomenon that has been examined as a potential control method for *H. virescens*. Sterile hybrid male offspring result from interspecific mating of *H. subflexa* females with *H. virescens* males (Laster, 1972), and hybrid females backcrossed to *H. virescens* males continue to produce infertile male offspring. The introduction of such sterile males into the environment was tested as a method for suppressing the *H. virescens* population (Proshold et al., 1983).

The contents of the pheromone gland of *H. subflexa* females have been examined in some detail (Teal et al., 1981; Klun et al., 1982; Heath et al., 1991; Teal and Tumlinson, 1997). The chemicals present in volatile collections included: (*Z*)11-hexadecenal (*Z*11–16:Ald), (*Z*)-9-hexadecenal (*Z*9–16:Ald), hexadecenal (16:Ald), (*Z*)-11-hexadecenyl acetate (*Z*11–16:Ac), (*Z*)-9-hexadecenyl acetate (*Z*9–16:Ac), (*Z*)-7-hexadecenyl acetate (*Z*7–16:Ac), (*Z*)-11-hexadecenol (*Z*11–16:OH), and (*Z*)-9-hexadecenol (*Z*9–16:OH) (Teal et al., 1981; Heath et al., 1991). Klun et al. (1982) reported the presence of small amounts of tetradecanal (14:Ald), (*Z*)-9-tetradecenal (*Z*9–14:Ald), (*Z*)-7-hexadecenal (*Z*7–16:Ald), and hexadecenyl acetate (16:Ac) in addition to those compounds reported by Teal et al. (1981), with the exception of Z9–16:OH. Comparisons with volatiles extracted from *H. virescens* pheromone glands revealed that *H. virescens* females did not manufacture any compounds that had an acetate terminal moiety (Roelofs et al., 1974; Tumlinson et al., 1975; Klun et al., 1980a; Pope et al., 1982; Vetter and Baker, 1983; Teal et al., 1986; Teal and Tumlinson, 1997). In addition, *H. virescens* females do not emit either *Z*9–16:OH or *Z*11–16:OH, even though both compounds can be found in gland extracts (Teal et al., 1986; Heath et al., 1991). It seems doubtful that *H. subflexa* females produce any of the shorter chain C_{14} aldehydes (14:Ald and *Z*9–14:Ald) emitted by *H. virescens* females as reported in the study of Klun et al. (1982). These compounds have not been found in any other analyses of *H. subflexa* female pheromone gland production (Teal et al., 1981; Heath et al., 1991). The compounds present in the pheromone glands of both *H. virescens* and *H. subflexa* females are summarized in Figure 1.

Initial field-trapping experiments revealed that most males were captured when *Z*11–16:Ald, *Z*9–16:Ald, *Z*11–16:Ac, *Z*9–16:Ac, and *Z*7–16:Ac were present on the pheromone lure (Teal et al., 1981). Subsequently, Heath et al. (1990) reported that *Z*11–16:OH was a critical component of the pheromone, required in combination with 16:Ald, *Z*11–16:Ald, *Z*9–16:Ald, *Z*9–16:Ac, and *Z*11–16:Ac,

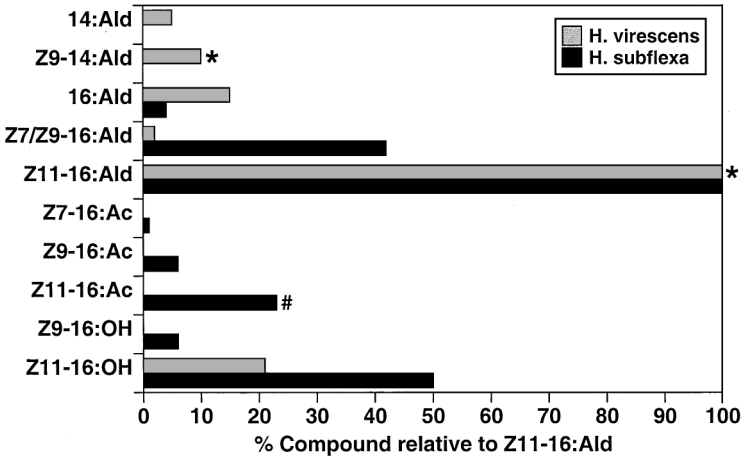


FIG. 1. A comparison of the compounds detected in the pheromone gland of *H. virescens* and *H. subflexa* females (data from Teal and Tumlinson, 1997). Female *H. subflexa* do not produce any of the shorter C_{14} aldehydes (14:Ald, *Z*9–14:Ald). Female *H. virescens* do not produce any of the C_{16} acetates including *Z*11–16:Ac (#), a potent antagonist of *H. virescens* males (Vickers and Baker, 1997). Compounds forming part of an attractive pheromonal blend for *H. virescens* males are indicated (*). While *Z*11–16:OH is present in the pheromone gland of *H. virescens* females, it does not appear to be emitted and utilized by conspecific males.

for attraction of males under field conditions. Because *H. virescens* and *H. subflexa* are sympatric throughout much of their range in the eastern United States, we predicted that males would exploit the differences in female gland emissions with behavioral requirements for distinct pheromonal blends under wind-tunnel conditions. The purpose of the current study was, therefore, to determine which volatile constituents of the female *H. subflexa* pheromone gland are essential for the attraction of conspecific males.

METHODS AND MATERIALS

Insects. A colony of *H. subflexa* was established in January 1999 from pupae and eggs sent to the University of Utah from North Carolina State University. This colony had been initiated from insects collected in the field during summer/fall of 1998. Neonate larvae were reared on a corn–soy blend diet (A. Sheck, personal communication) in individual 30-ml medicine cups fitted with cardboard lids (WLM Inc.). Once the larvae had pupated, they were removed from the cups,

and pupae were separated into males and females. Male pupae for experimental use were placed in an environmental chamber (Percival Scientific, Boone, Iowa, USA) at 25°C, 60% relative humidity, and 14L:10D reversed light cycle and allowed to eclose. Males were aged daily and cohorts of aged males were keep in plastic containers. Males between the ages of 3 and 10 days were utilized in wind-tunnel experiments. On the day of experimentation, males were placed into 5- × 6-cm (W × H) cylindrical wire screen cages. The cages were inverted to prevent escape from the open end and were placed into a larger plastic container. The container was then returned to the environmental chamber prior to the onset of scotophase before being moved to the darkened wind tunnel room during the third hour of scotophase. Individual cages were removed from the container and placed on one side of the wind tunnel. Males were afforded a period of at least one hour to acclimate to the conditions in the wind tunnel prior to being flown between 3 and 6 hr of scotophase. Individual males were released by inverting their cage on a platform, allowing each male to take flight from the now open cage end. The platform intersected the pheromone plume, being positioned in the horizontal center of the wind tunnel, 50 cm from the downwind end and 19 cm above the wind-tunnel floor.

Wind Tunnel. The working section of the wind tunnel was 2.5 × 1.14 × 1.14 m (L × H × W). Wind speed varied between 0.45 and 0.61 m/sec. Temperature in the wind-tunnel room varied between 21.1 and 22.2°C with ambient relative humidity of 12.8–55.2%. The wind tunnel was illuminated by a single red incandescent light bulb above the downwind end and a single white incandescent bulb at the upwind end, both controlled by rheostats. The plume was vented to the exterior of the building at the downwind end of the wind tunnel via a large exhaust duct.

Pheromone Components. Stock solutions of individual compounds were maintained at −20°C. Dilutions were made volumetrically in hexane until a concentration of 100 ng/μl was achieved for *Z*11–16:Ald and *Z*9–16:Ald, and dilutions of 10 ng/μl were obtained for *Z*9–16:Ald, *Z*11–16:OH, *Z*11–16:Ac, and *Z*9–14:Ald (the latter compound was also diluted to 1 ng/μl). The purity of each solution was established by injection of a small volume onto a Shimadzu GC 17A gas chromatograph equipped with a 30-m DB-5 capillary column (0.25 mm ID). All compounds were >95% pure. Each blend was created by admixing the appropriate volumes of the required single compounds directly onto a circular filter paper disk (Whatman No. 4, 1-cm diam.). All compounds in a given blend were loaded in a ratio with respect to *Z*11–16:Ald, which was always present at 1000 ng (denoted 1). For example, to achieve a ratio of 1:0.5 *Z*11–16:Ald and *Z*9–16:Ald (or 50% *Z*9–16:Ald), 500 ng *Z*9–16:Ald was added onto a filter paper disk already impregnated with 1000 ng *Z*11–16:Ald. The hexane was allowed to evaporate in the fume hood. The disk was held by a small alligator clip mounted on a wire post with a heavy rubber cork base. The holder, when complete with the filter paper

TABLE 1. PHEROMONE BLEND MIXTURES TESTED FOR ATTRACTION OF *H. subflexa* MALES

	Compound				
Treatment	*Z*11–16:Ald	*Z*9–16:Ald	*Z*11–16:OH	*Z*11–16:Ac	*Z*9–14:Ald
Experiment 1					
1	1[a]	0.5	0.1	0	
2	1	0.5	0	0.1	
3	1	0.5	0.1	0.1	
4	1	0	0.1	0.1	
Experiment 2					
1	1	0.5	0.1		
2	1	0.1	0.1		
3	1	0.01	0.1		
4	1	0	0.1		
Experiment 3					
1	1	0.5	0.5		
2	1	0.5	0.1		
3	1	0.5	0.01		
4	1	0.5	0		
Experiment 4					
1	1	0.5	0.1		0
2	1	0	0.1		0.005
3	1	0	0.1		0.05
4	1	0	0.1		0.5

[a] A ratio of 1 indicates that the compound was loaded at a dosage of 1000 ng.

disk, measured 24.5 cm tall. The distance between the odor source and takeoff platform in the wind tunnel was 1 m.

Experimental Design. Treatments were presented in a randomized complete block design. Groups of three to five males were flown to each treatment on any given day. The treatments presented in each experiment are shown in Table 1.

Data Analysis. The behavioral responses of males were categorized according to the following sequential series: (1) Flight: male exited cage and initiated flight in the wind tunnel. (2) Lock-on: male exhibited narrow turns perpendicular to wind line in the area of the take off platform at height of pheromone plume. (3) Upwind: male proceeded upwind in characteristic zig-zag flight at height of pheromone plume. The distance of flight toward the source was noted. Random upwind flight lacking characteristic orientation behaviors and outside the vicinity of the plume (along walls or ceiling) was not included as upwind flight. (4) Close approach: oriented upwind flight continued to within 5cm of pheromone source. (5) Source: male landed on filter paper source or clip holding source.

Percentages of males performing each behavior were calculated and subjected to a χ^2 2 × 2 test of independence with Yates' correction (Steel and Torrie, 1961). Significance was determined at the $P < 0.05$ level.

RESULTS

The series of blends tested and ratios utilized in experiment 1 (Table 1) was based upon previous field-trapping data and the known constituents of female *H. subflexa* pheromone gland constituents (Figure 1) (Teal et al., 1981; Klun et al., 1982; Heath et al., 1990, 1991; Teal and Tumlinson, 1997). The first experiment (Figure 2) clearly demonstrated that both *Z*9–16:Ald and *Z*11–16:OH were essential components of the pheromone blend. The two blends that lacked either of these compounds elicited very low levels of upwind flight, and virtually no males located the source in response to either blend. The addition of *Z*11–16:Ac to a blend containing *Z*11–16:Ald, *Z*9–16:Ald, and *Z*11–16:OH increased the number of males contacting the source, but this difference was not significantly greater compared to the blend in which this compound was omitted.

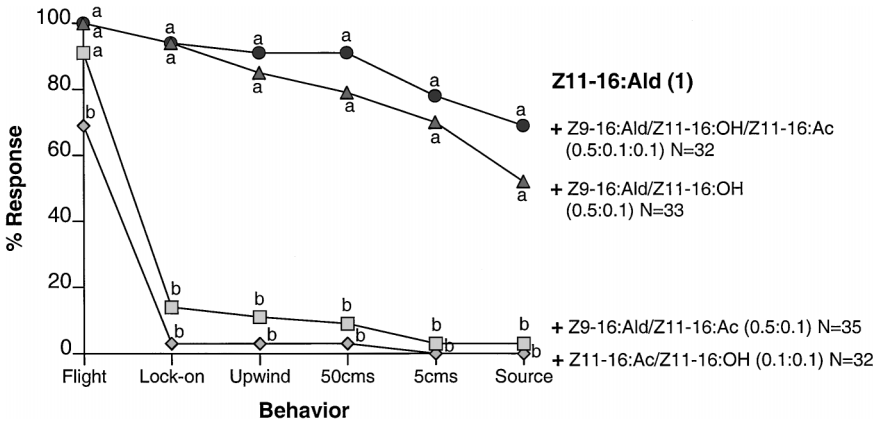


FIG. 2. Test of a series of blends on behavioral responses of *H. subflexa* males. The performance of each male in a sequential series of behaviors was noted: male took flight (Flight); male oriented to the pheromone plume (Lock-on), male initiated upwind flight (Upwind), upwind flight sustained to within 50 cm of source (50 cms) or 5 cm (5 cms), male lands on odor source (Source). The ratios utilized in this first experiment were based upon the data from female gland analyses (Teal and Tumlinson, 1997, summarized in Figure 1). *Z*11–16:Ald was held constant in each test mixture, with other compounds varying as indicated. Both *Z*9–16:Ald and *Z*11–16:OH were required to elicit significant and high levels of upwind flight and source location. Symbols in the same behavioral category having no letters in common are significantly different according to a χ^2 2 × 2 test of independence with Yates' correction. ($P < 0.05$)

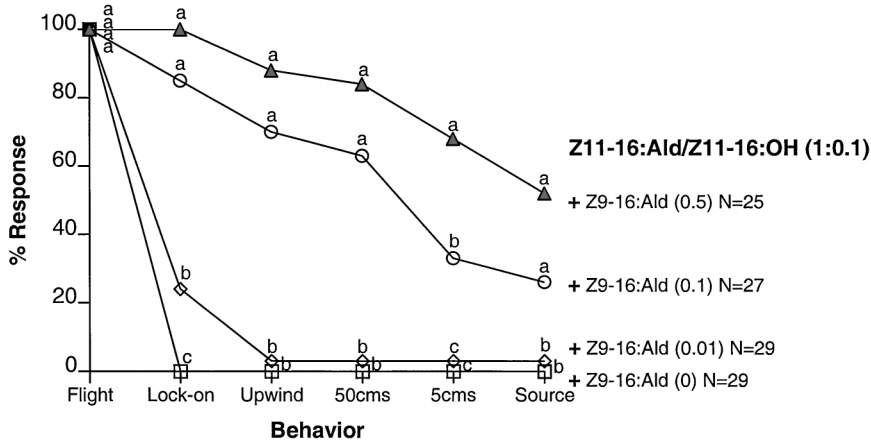


FIG. 3. Behavioral responses of *H. subflexa* males to a series of blends that contained varying dosages of *Z*9–16:Ald relative to constant dosages of *Z*11–16:Ald (1000 ng) and *Z*11–16:OH (100 ng). Significant levels of upwind flight and source location were attained only when the ratio of *Z*9–16:Ald was 0.1 (100 ng) or greater with respect to *Z*11–16:Ald. Behavioral and statistical details as for Figure 2.

The initial observation of the importance of *Z*9–16:Ald was confirmed in a second experiment (Table 1, Figure 3). When *Z*11–16:Ald and *Z*11–16:OH were held constant at a ratio of 1:0.1, males failed to respond to the blend lacking *Z*9–16:Ald. Upwind flight and source location were not significantly enhanced by the presence of a small amount of *Z*9–16:Ald (0.01 or 1%). Significant levels in all orientation categories were achieved with the addition of 10% *Z*9–16: Ald. When the amount of *Z*9–16:Ald was increased to 50%, more males reached within 5 cm of the source, but there was no statistical difference between 10% and 50% Z9–16:Ald treatments for males contacting the odor source.

In experiment 3, the ratio of *Z*11–16: Ald to *Z*9–16:Ald was held constant at 1:0.5 and the amount of *Z*11–16:OH was varied (Table 1, Figure 4). Males showed no signs of attraction to blends that lacked *Z*11–16:OH, confirming the result from experiment 1. However, addition of a small amount of *Z*11–16:OH improved attraction. Source location was improved to 69% when only 1% *Z*11–16:OH was added to the binary mixture of *Z*11–16:Ald and *Z*9–16:Ald. Increasing the amount of *Z*11–16:OH (10%, 50%) neither enhanced nor inhibited upwind flight and source location compared to the Z11–16: Ald treatment.

In experiment 4, responses of males to blends in which *Z*9–16:Ald had been replaced with varying dosages of *Z*9–14:Ald were tested (Table 1, Figure 5). Males were not attracted by blends in which this substitution had been effected.

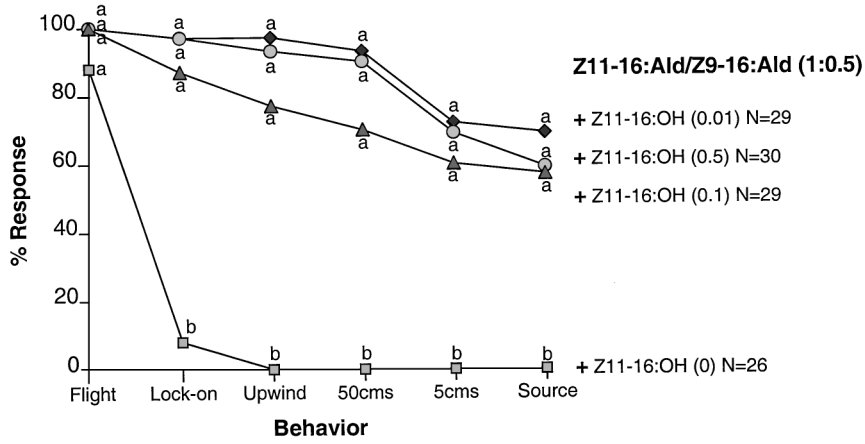


FIG. 4. Behavioral responses of *H. subflexa* males to a series of blends that contained varying dosages of *Z*11–16:OH relative to constant dosages of *Z*11–16:Ald (1000 ng) and *Z*9–16:Ald (500 ng). Males did not respond to a blend in which *Z*11–16:OH was excluded. However, males were sensitive to the presence of this compound in the mixture and responded at low dosages (10 ng, ratio 0.01). Increasing the dosage (100 ng, ratio 0.1; or 500 ng, ratio 0.5) neither elevated nor diminished male response levels. Behavioral and statistical details as for Figure 2.

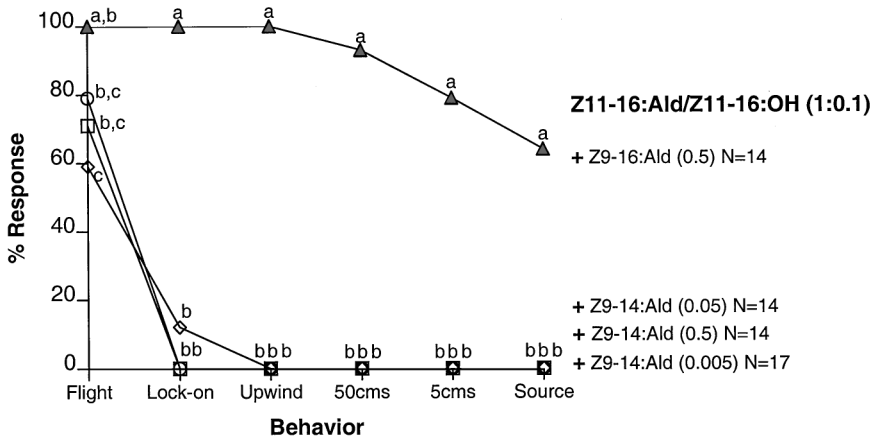


FIG. 5. Effects of replacing *Z*9–16:Ald with varying dosages of *Z*9–14:Ald on behavioral responses of *H. subflexa* males. Amounts of *Z*11–16:Ald and *Z*11–16:OH were held constant at a ratio of 1:0.1. Males were not attracted to any blend in which *Z*9–16:Ald was replaced with *Z*9–14:Ald, the secondary component of the closely-related congeneric species, *H. virescens*. Behavioral and statistical details as for Figure 2.

DISCUSSION

Male *H. subflexa* were attracted to filter paper sources impregnated with a mixture containing *Z*11–16:Ald, *Z*9–16:Ald (10% or greater), and *Z*11–16:OH (1% or greater). These compounds have previously been identified in pheromone gland extracts from *H. subflexa* females (Teal et al., 1981; Klun et al., 1982; Heath et al., 1991; Teal and Tumlinson, 1997). At least 10% *Z*9–16:Ald was required to elicit significant levels of upwind flight and source location (Figure 3), whereas dosages as low as 1% *Z*11–16:OH (Figure 4), exceeded the behavioral threshold for this compound. Field-trapping studies led Teal et al. (1981) to conclude that male *H. subflexa* did not require the presence of *Z*11–16:OH, but a subsequent field study (Heath et al., 1990) revealed that this compound was essential for attraction. Our results concur with those of Heath et al. (1990) in clearly demonstrating that male *H. subflexa* require the presence of *Z*11–16:OH in the pheromone blend. Furthermore, males appeared to be extremely sensitive to the presence of this compound. Addition of as little as 10 ng (1%) *Z*11–16:OH relative to *Z*11–16:Ald (1000 ng) resulted in 68% upwind flight and source location compared to 0% source location when *Z*11–16:OH was excluded from the test blend (Figure 4). The threshold for behavioral responses to this compound is, therefore, below the 10 ng dosage tested in these experiments. No significant improvement or deterioration in the ability of males to locate the pheromone source was noted as the dosage of *Z*11–16:OH was increased to 500 ng (50%). The precise release rates of the test compounds from the filter paper substrate utilized in these experiments, however, are not known. Heath et al. (1990) found that release ratios in excess of 3.5% *Z*11–16:OH resulted in decreased trap capture of *H. subflexa* males. The formulations used in that study were based upon known release rates of compounds from rubber septa (Heath et al., 1986).

The composition of the *H. subflexa* pheromonal mixture is interesting when potential interactions with other species are considered. Avoidance of attraction between *H. subflexa* and *H. virescens* has obvious biological significance given the ability of these two species to hybridize and the reduced fitness of the offspring (in the form of male sterility) (Laster, 1972). The apparent lack of *Z*9–14:Ald in the *H. subflexa* pheromone gland (Teal et al., 1981; Heath et al., 1991) would prevent the attraction of *H. virescens* males, which require the presence of this compound in small amounts (Vickers et al., 1991). Even if female *H. subflexa* were to produce 0.5% of this compound relative to *Z*11–16:Ald, as reported by Klun et al. (1982), the additional presence of *Z*11–16:Ac [63% reported by Klun et al. (1982), 41% by Teal et al. (1986)], which has been shown to be a potent inhibitor of *H. virescens* males even at a loading of 1% (Vickers and Baker, 1997), would prevent the cross-attraction of *H. virescens* males. It is unlikely that male *H. subflexa* would be attracted to *H. virescens* females for two reasons: (1) the small amount of *Z*9–16:Ald relative to *Z*11–16:Ald produced by *H. virescens*

females [1.6% reported by Klun et al. (1980), 1.7% by Teal et al. (1986)] may be insufficient to attract *H. subflexa* males because the upwind flight threshold for this compound is between 1% and 10% (Figure 3), and (2) even though *Z*11–16:OH can be extracted from female *H. virescens* pheromone glands (Klun et al., 1979, 1980a; Teal et al., 1986), it is not released by calling females (Pope et al., 1982; Teal et al., 1986; Heath et al., 1991). Indeed, field results have indicated that no cross-attraction occurs between traps baited with synthetic pheromone blends of *H. virescens*, *H. subflexa*, and a third related North American species, *Helicoverpa zea* (Klun et al., 1982).

The presence of *Z*11–16:OH in female *H. virescens* pheromone glands has been debated for a number of years. Klun et al. (1980a) first reported the presence of *Z*11–16:OH, but other studies were unable to isolate this compound (Pope et al., 1982). Teal et al. (1986) identified *Z*11–16:OH in gland extracts but did not detect it in volatile emissions from calling females or extruded pheromone glands. According to biosynthetic schemes, *Z*11–16:OH is a precursor of the major pheromone component and is rapidly converted to *Z*11–16:Ald by an alcohol oxidase in the pheromone glands of both *H. virescens* and *H. zea* (Teal and Tumlinson, 1986, 1987). Vetter and Baker (1983) demonstrated that the addition of *Z*11–16:OH to an attractive blend was not deleterious to *H. virescens* male behavior until loading exceeded 3%, with lower concentrations having no observable effect. In other studies, small amounts of *Z*11–16:OH were shown to increase trap catches of *H. virescens* males (Sparks et al., 1979; Ramaswamy et al., 1985). Recent results indicate that *Z*11–16:OH does not have a clear inhibitory effect on males tested from laboratory populations (N. J. Vickers, unpublished observations).

An obvious possibility to further diminish the likelihood of interspecific attraction is the use of *Z*9–14:Ald released by *H. virescens* females as an antagonist by *H. subflexa* males. However, preliminary experiments have revealed that *H. subflexa* males are neither attracted nor repelled when this compound is combined with an otherwise attractive blend (N. J. Vickers, unpublished observations). In addition, *Z*9–14:Ald cannot be used in place of *Z*9–16:Ald at any dosage tested thus far (Figure 5). This contrasts with *H. zea* where substitution of the usual secondary component, *Z*9–16:Ald, with small amounts of *Z*9–14:Ald can be achieved without significantly impacting the levels of upwind flight and source location (Vickers et al., 1991). Despite the current lack of evidence for a clear antagonist, it is apparent from on-going experimentation involving *H. subflexa*/*H. virescens* hybrids in our laboratory that mating between *H. virescens* females and *H. subflexa* males is difficult to achieve (in contrast to the reciprocal cross), perhaps indicating other pre- or postmating reproductive isolation mechanisms.

Another possible interspecific interaction might occur between *H. subflexa* and *H. zea*. Although there is no evidence for hybridization with this more distantly related heliothine moth, male *H. zea* require a mixture of two compounds, *Z*11–16:Ald and *Z*9–16:Ald, both of which are present in the *H. subflexa* pheromone

gland. However, *H. zea* males are antagonized by the presence of two other compounds present in the pheromone blend emitted by *H. subflexa* females, *Z*11–16:Ac (Fadamiro et al., 1999) and *Z*11–16:OH (Teal et al., 1984; Quero and Baker, 1999). Conversely, male *H. subflexa* are not attracted to *H. zea* females because they do not emit *Z*11–16:OH (Klun et al., 1979, 1980b; Pope et al., 1984) even though this compound is abundantly present in pheromone gland extracts (see above, Teal et al., 1984, 1989).

There are several other compounds present in the pheromone gland of *H. subflexa* females that were not tested in this series of experiments. These include 16:Ald, *Z*7–16:Ac, *Z*9–16:Ac, and *Z*9–16:OH (Teal et al., 1981). These compounds may play important roles in the pheromone-mediated behavior of *H. subflexa* males, and their potential behavioral effects will be examined in future experiments. The current study reveals the minimum pheromonal requirement by *H. subflexa* males to be *Z*11–16:Ald, *Z*9–16:Ald, and *Z*11–16:OH. Behavioral observations suggest an optimal ratio of 1:0.5:0.01–0.1. The effects of *Z*11–16:Ac upon *H. subflexa* male behavior remains somewhat ambiguous. It may play a pivotal role under field conditions where the background chemical "noise" from other species may have a significant impact upon blend discrimination.

Molecular analysis of heliothine moth phylogeny utilizing nuclear DNA (Cho et al., 1995; Fang et al., 1997) and morphologically based surveys (Mitter et al., 1993) have revealed that *H. virescens* and *H. subflexa* belong within the monophyletic *virescens* group. The requirement by males of these two species for pheromone blends that differ markedly in their secondary component characteristics provides an excellent opportunity to explore the underlying changes in the male olfactory system accompanying this divergence in blend preferences.

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VARIATION IN A FEMALE SEXUAL ATTRACTIVENESS PHEROMONE CONTROLS MALE MATE CHOICE IN GARTER SNAKES

MICHAEL P. LEMASTER* and ROBERT T. MASON

Department of Zoology
Oregon State University
Corvallis, Oregon 97331-2914

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Abstract—Male red-sided garter snakes (*Thamnophis sirtalis parietalis*) display a courtship preference for larger females during the breeding season. Utilizing behavioral experiments and chemical analyses, we tested the hypothesis that males can discriminate among females of varying size solely by means of the sexual attractiveness pheromone, a previously characterized sex pheromone composed of a homologous series of long-chain saturated and ω-9 *cis*-unsaturated methyl ketones contained in the skin lipids of females. When presented with skin lipid extracts from large and small females, a greater proportion of males displayed courtship behaviors to large female extracts. This demonstrates that there is an intrinsic property of the female skin lipids that allows males to differentiate among large and small females. Analysis of the sexual attractiveness pheromone revealed that the necessary variation exists for this pheromone to function as a reliable indicator to males of female body size. Specifically, we observed a strong correlation between female snout–vent length and the relative concentration of saturated and ω-9 *cis*-unsaturated methyl ketones composing the pheromone; smaller females expressed pheromone profiles higher in saturated methyl ketones, while larger females expressed pheromone profiles dominated by unsaturated methyl ketones. The results of this study suggest that male red-sided garter snakes utilize compositional variation in the female sexual attractiveness pheromone to differentiate among potential mates of varying size.

Key Words—Mate choice, sexual attractiveness pheromone, qualitative variation, methyl ketones, red-sided garter snake, *Thamnophis sirtalis parietalis*.

* To whom correspondence should be addressed. E-mail: lemastem@bcc.orst.edu

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INTRODUCTION

In many vertebrate species, individuals of one or both sexes actively select among potential mates during the breeding season in an effort to optimize their reproductive success (Andersson, 1994). Individuals often discriminate among potential mates based on variation in the expression of a particular morphological trait. For example, females of some bird species differentiate among males based on variation in visual traits [e.g., color intensity (Hill, 1990), tail length (Møller, 1988)], whereas female frogs may utilize variation in auditory traits [e.g., call rate (Sullivan, 1983), call pitch (Ryan, 1980)]. Chemical traits, such as pheromones (Karlson and Lüscher, 1959), represent an additional type of morphological trait by which potential mates might be evaluated.

The occurrence of chemically mediated mate choice is relatively common in insects, where chemical communication is considered the dominant modality for controlling reproductive behavior in many species (Bell and Cardé, 1984). Unlike insects, however, few studies have documented chemically mediated mate choice in vertebrates (e.g., Verrell, 1985; Reece-Engel, 1988; Martín and López, 2000). This paucity of studies is primarily due to the multicomponent sensory nature of vertebrates where a combination of sensory inputs (e.g., visual, tactile, chemical) is often responsible for mediating a particular behavior (Albone, 1984). Such diversification makes it difficult to establish effective bioassays for measuring behavioral responses of individuals to isolated chemical cues to determine to what extent such cues are utilized.

Snakes offer an excellent model system in which to investigate the chemical mediation of mate choice in vertebrates. More than most vertebrates, snakes rely extensively upon the production and perception of specific sex pheromones for the coordination of reproductive behavior (reviewed in Mason, 1992; Mason et al., 1998). Hence, there is an increased probability that mechanisms evolved to select among potential mates will be chemical in nature [mate choice in snakes (Hawley and Aleksiuk, 1976; Luiselli, 1996)]. In addition, snakes are much like insects in that they respond to isolated chemical cues with stereotypical, robust behaviors (Brown and MacLean, 1983; Mason and Crews, 1985). Thus, behavioral bioassays can be constructed with relative ease to test the responses of snakes to chemical cues from potential mates when presented in isolation.

Annual aggregations of red-sided garter snakes (*Thamnophis sirtalis parietalis*) at underground hibernacula in Manitoba, Canada, are unique natural phenomena, representing the highest concentrations of snakes in the world (Gregory, 1984). Mating in these populations occurs directly at the hibernacula following spring emergence, with virtually all females [>95% (Garstka et al., 1982)] mating within one day of emerging from winter dormancy. During this period, it is not uncommon to observe 10–100 males courting a newly emerged female, forming what are termed "mating balls" (Gregory, 1974, 1977). Males do not randomly associate

with females, but instead display a courtship preference for larger females (Hawley and Aleksiuk, 1976; Shine et al., 2001).

It has been hypothesized that the cue utilized by male red-sided garter snakes to differentiate among potential mates is the sexual attractiveness pheromone (Garstka et al., 1982; Gregory, 1984; Mason, 1992). Composed of a homologous series of saturated and ω-9 *cis*-unsaturated methyl ketones (Mason et al., 1989, 1990), this pheromone is sequestered in the skin lipids of females during the breeding season and is primarily responsible for eliciting male courtship behavior (Noble, 1937; Garstka et al., 1982). Indeed, if a male does not detect this pheromone on the dorsal surface of a female, then courtship behavior will not be initiated and mating will not occur (Mason, 1993). Thus, variation in the expression of the sexual attractiveness pheromone by females of varying size may directly influence male courtship behavior, leading to the mating preference observed in this species.

Here, we report a study designed to investigate the role of the female sexual attractiveness pheromone in mediating male mate choice in the red-sided garter snake. Our two major aims in this study were: (1) to determine whether males can discriminate among females of varying size based solely on chemical cues in the skin lipids, and (2) to evaluate whether the sexual attractiveness pheromone contains the necessary variation to function in male mate choice. To this end, we conducted behavioral experiments testing the courtship response of males to large and small females and skin lipid extracts from large and small females. Further, we collected pheromone samples from females of varying size and examined the quantity and quality of pheromone expressed by individual females.

METHODS AND MATERIALS

Study Population

Red-sided garter snakes utilized for this study were captured at a field site near the community of Inwood in the Interlake region of Manitoba, Canada (50°31.58′N; 97°29.71′W) during May 2000. This site is located at an abandoned gravel quarry containing a single overwintering hibernaculum possessing in excess of 10,000 red-sided garter snakes during the winter months (R. T. Mason, unpublished data). Adult females in this population attain an average snout–vent length (SVL) of 55–60 cm and a mean mass of 70–85 g, while adult males are much smaller, attaining an average SVL of 45–50 cm and a mean mass of 35–40 g (Shine et al., 1999).

Behavioral Experiments

Experimental Animals. Unmated large female adult red-sided garter snakes (SVL $>$ 60.0 cm; $N = 35$) and small female adult red-sided garter snakes

(SVL < 50.0 cm; $N = 35$) were collected at the hibernaculum immediately upon emergence. We established the SVL criteria for large and small females based on the results of Hawley and Aleksiuk (1976), who found that female red-sided garter snakes with SVL > 60 cm were twice as likely to be courted by males than females with SVL < 50 cm. Adult males ($N = 380$; SVL $\pm$ SD $= 52.4 \pm 2.54$) were collected from naturally occurring mating balls located around the hibernaculum. We focused our collection on larger males (SVL > 50.0 cm), as larger males show an increased ability to differentiate between females based on size compared to smaller males in this species (Shine et al., 2001). Animals were segregated by sex and size in cloth bags and held at ambient temperatures until testing, after which the animals were returned to the hibernaculum and released.

Testing Conditions. Behavioral experiments were carried out in outdoor arenas measuring $1 \times 1 \times 1$ m and constructed of nylon fabric attached to metal posts inserted in the ground. Arenas of this type do not appear to alter male reproductive behaviors compared to the behaviors observed in the wild, and as a result such arenas have been used extensively in the study of snake reproductive ecology (e.g., Mason and Crews, 1985; Shine et al., 1999; Moore et al., 2000). To avoid any confounding effects due to weather conditions, an array of four arenas was established, allowing for groups of trials to be carried out simultaneously. Testing days and times of day (10:00 hr to 16:00 hr) were chosen so that all experiments were conducted under similar ambient conditions (mostly sunny skies with light winds and temperatures of 15–20°C), which correspond with conditions when red-sided garter snakes are most active (M. P. LeMaster, personal observation).

Experiment 1. To verify that males in our study population exhibited a courtship preference for larger females, we first tested the behavioral response of males to large and small females using a simultaneous choice test design (e.g., Mason and Crews, 1985). Briefly, we introduced 10 randomly chosen males into an arena and allowed them to acclimatize for 5 min. We then placed into each arena simultaneously a large and small female. The snakes were allowed to interact undisturbed for 5 min, after which time the number of males actively courting each female size was recorded. A total of 16 tests were performed with unique male and female snakes used in each trial.

Male courtship behavior was assessed by using an ethogram of male garter snake mating behavior (Table 1). Similar ethograms are routinely used in field and laboratory studies with garter snakes (e.g., Crews et al., 1984; Moore et al., 2000). To prevent females from mating during the trials, a condition that drastically reduces further male courtship advances (Garstka et al., 1982), we placed adhesive tape across the female cloaca. The tape was then removed immediately upon completion of each trial.

Experiment 2. This experiment utilized behavioral trials to determine whether chemical cues contained within the skin lipids of females are sufficient for male mate discrimination. For each trial, we introduced 10 randomly chosen males into

TABLE 1. ETHOGRAM OF COURTSHIP BEHAVIOR FOR MALE RED-SIDED GARTER SNAKES, *Thamnophis sirtalis parietalis*[a]

Courtship score	Description of behavior
1.0	Male investigates female with increasing tongue-flick rate
2.0	Male chin-rubs dorsal surface of female and aligns body with female
3.0	Male actively tail searches and attempts cloacal apposition with female
4.0	Male copulates with female

[a] Modified from Crews et al. (1984). A behavioral score of 2.0 or greater only occurs in a reproductive context and is, therefore, indicative of sexual behavior for males of this species.

an arena and allowed them to acclimatize for 5 min. We then randomly placed in with the males one of four treatments: (1) a small female, (2) a large female, (3) filter paper containing a small female skin lipid sample, or (4) filter paper containing a large female skin lipid sample. Male snakes were allowed to interact with the female or filter paper undisturbed for 5 min, after which the number of males actively courting the treatment was recorded. For filter paper treatments, a male had to be rapidly tongue-flicking and chin-rubbing the filter paper for it to be considered actively courting. A total of 38 trials were performed using small ($N = 9$) and large ($N = 9$) female snakes and skin lipid samples from small ($N = 10$) and large ($N = 10$) female snakes. Unique males and females were used in each behavioral trial.

Skin lipid samples were collected from large and small females by rubbing down the dorsal surface of the donor snake with filter paper wetted with 100% hexane. Care was taken to prevent contamination from cloacal gland secretions (Mason et al., 1989). For each skin lipid sample collected, a unique female was used that was independent of females used in the behavioral trials. To control for surface area differences in the two groups of females, we rubbed down the length of the large females once with the filter paper and rubbed down the length of the small females three times. We established this protocol based on prior knowledge that hexane extracts from large females yield three times the amount of skin lipids compared to small females and that multiple rubbing of a female does not affect male behavior towards the subsequent skin lipids extracted (M. P. LeMaster, unpublished data). To verify that similar quantities of skin lipids were laid down for each female size, we compared the average skin lipid amount laid down on the filter papers for each female size upon completion of the trials [skin lipids (milligrams) = weight of treated filter paper − weight of blank filter paper].

Chemical Analysis

Pheromone Collection. Adult, sexually attractive female red-sided garter snakes of varying size (SVL range 48.0–74.2 cm; $N = 20$) were collected

immediately upon spring emergence from the hibernaculum. The size range of females collected corresponded with the size range of females used in our behavioral experiments. The animals were killed with an overdose of brevital sodium. Each snake was then placed dorsal side down in a 500-ml glass beaker and covered with 25–50 ml of 100% hexane (C_6H_{12}) for 12 hr (Mason et al., 1989, 1990). Care was taken to keep the head and cloaca out of the hexane to avoid possible contamination by internal body fluids. After removal of the animals, the excess solvent was removed under reduced pressure by rotoevaporation at 35°C. The resulting residues were weighed on a digital scale (Mettler AT400), resuspended in fresh hexane (1–2 ml), and sealed in 9-ml glass vials with polyethylene-lined caps for storage at −20°C.

To isolate the methyl ketones composing the sexual attractiveness pheromone, we fractionated the skin lipid extracts using column chromatography as described by Mason et al. (1989). Briefly, we loaded the skin lipid extracts onto glass columns (350 mm long × 22 mm ID) packed with alumina (activity III) and eluted the columns with hexane and ethyl ether ($C_4H_{10}O$) solutions of increasing polarity. For each sample, the fraction containing the appropriate methyl ketones [fraction 5 (Mason et al., 1989)] was collected, and the excess solvent was removed by rotoevaporation (35°C). The resulting methyl ketone residues were weighed on a digital scale (Mettler AT400) and resuspended in 1 ml fresh hexane. Samples were then placed in 9-ml glass vials with polyethylene-lined caps and stored at −20°C until further analysis.

Pheromone Analysis. Multicomponent pheromones, such as the sexual attractiveness pheromone of the red-sided garter snake, can show variation in both the quantity and quality of pheromone expressed. To examine variation in the quantity of pheromone expressed by female snakes of varying size, we calculated the amount of methyl ketones expressed per unit skin surface area (micrograms per square centimeter) for individual females. This was accomplished by dividing the weight of the isolated methyl ketone residues extracted from a female by the total skin surface area of the female. A general index of skin surface area for each female was determined by multiplying the snout–vent length of a female by its circumference at mid-body (Mason et al., 1990).

To examine variation in the quality of pheromone expressed by females, we determined the number of unique methyl ketones expressed by individual females and calculated the relative contributions of saturated *versus* monounsaturated methyl ketones to the individual pheromone profiles. The methyl ketones present in the pheromone extracts were identified utilizing a Hewlett Packard 5890 Series II gas chromatograph fitted with a split injector (280°C) and a Hewlett Packard 5971 Series mass selective detector. Aliquots (1 μl) of the methyl ketone fractions were injected onto a fused-silica capillary column (HP-1; 12 m × 0.22 mm ID; Hewlett Packard) with helium as the carrier gas (5 cm/sec). Oven temperature was initially held at 70°C for 1 min, increased to 210°C at 30°C/min, held at 210°C for 1 min,

increased to 310°C at 5°C/min, and finally held at 310°C for 5 min. Once the methyl ketones were identified, we calculated the relative concentrations of saturated and monounsaturated methyl ketones in each sample by using peak integration [e.g., percent relative concentration of saturated methyl ketones = (area under saturated methyl ketone peaks/total area of all methyl ketone peaks) × 100]. Identification of compounds and peak areas were determined utilizing ChemStation software (Version B.02.05; Hewlett Packard) interfaced with the gas chromatograph/mass spectrometer.

Statistics

Statistical analyses were performed using Jandel SigmaStat Version 2.0 software package (Jandel Corporation). The numbers of males courting large females compared to small females when presented simultaneously were examined by using χ^2 analyses. Differences in the proportion of males courting large and small females and large and small female skin lipid samples were initially analyzed by using a χ^2 test of proportions, with Tukey-type multiple comparison tests then used to perform pairwise comparisons among the four treatments (Zar, 1984). Finally, the relationship between the size of the female (SVL) and various quantitative and qualitative measures of pheromone expression were examined utilizing Pearson product moment correlation tests (Sokal and Rohlf, 1995). Level of significance for each test was set at $P < 0.05$.

RESULTS

Behavioral Experiments

Experiment 1. The average SVL ± SD of small females used in the simultaneous choice tests was 46.2 ± 2.70 cm, whereas the average SVL ± SD for large females was 63.0 ± 2.57 cm. This resulted in an average ± SD difference between small and large females presented to males during individual trials of 16.84 ± 3.93 cm. Over the 16 trials conducted, a greater proportion of males was observed courting the larger female over the smaller female following the 5-min interaction period ($\chi^2 = 56.47$, 1 *df*, $P < 0.001$; 64.3% of males courted large females vs. 11.9% of males courting small females) (Figure 1). We pooled the data for analysis after a heterogeneity χ^2 test showed no significant difference in male courtship preference among the individual trials conducted ($\chi^2 = 7.09$, 15 *df*, $P > 0.5$).

Experiment 2. When presented with the four treatments, male garter snakes responded with stereotypical courtship behaviors including increased tongue-flick rate, chin rubbing along the dorsum of the female (or filter paper), and body alignment with the female (or edge of filter paper). Overall, we observed significant variation in the proportion of males actively courting the various treatments

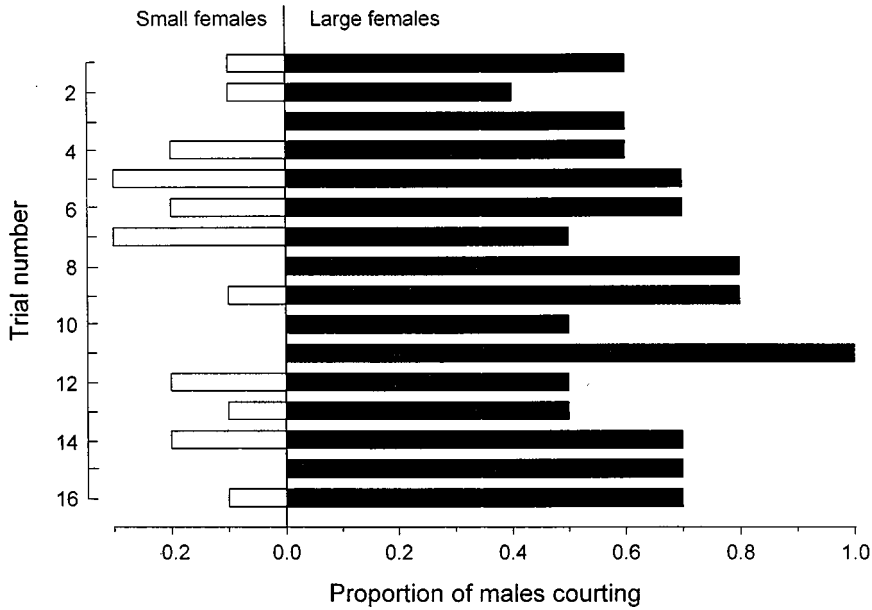


FIG. 1. Results of simultaneous choice tests in which a large and a small female red-sided garter snake (*Thamnophis sirtalis parietalis*) were placed in an outdoor arena with 10 courting males. After 5 min, the number of males that courted either female was recorded.

after the 5-min interaction period ($\chi^2 = 66.48$, 3 *df*, $P < 0.001$; Figure 2). Pairwise analysis revealed that a significantly greater proportion of males directed courtship behavior to large females and filter paper containing large female skin lipids than to small females and filter paper containing small female skin lipids (multiple comparison tests; $q = 10.4$–19.4; $P < 0.001$ for all tests). However, little difference was observed in the proportion of males displaying courtship behavior to large females and large female skin lipid extracts (multiple comparison test; $q = 3.23$; $P > 0.10$) and to small females and small female skin lipid extracts (Multiple comparison test; $q = 2.59$; $P > 0.20$).

When we examined the amount of skin lipids placed on the treated filter papers, we found no difference between filter papers containing large female skin lipid samples and filter papers containing small female skin lipid samples (large female average $\pm$ SD $= 3.0 \pm 2.4$ mg, small female average $\pm$ SD $= 3.2 \pm 2.0$ mg; ANOVA, $F_{1,18} = 0.041$, $P = 0.842$). In addition, there was no difference in SVL $\pm$ SD between large females presented to males and large female skin lipid donors (test female $= 66.1 \pm 3.63$ cm, donor female $= 66.7 \pm 4.66$ cm; t test: $t = 0.308$, $P = 0.762$) or between small females and small female skin lipid

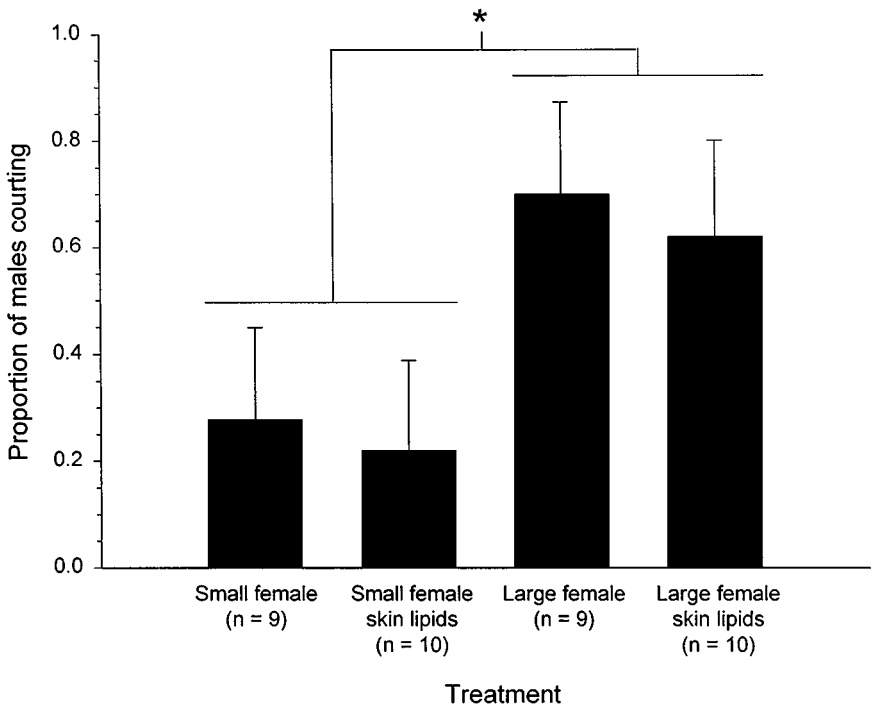


FIG. 2. Proportion (mean ± SD) of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) displaying courtship behavior to large and small females and skin lipid samples from large and small females.

donors (test female = 46.7 ± 4.60 cm, donor female = 44.2 ± 3.16 cm; t test: $t = 1.379$, $P = 0.186$).

Pheromone Analysis

Pheromone Quantity. The hexane extractions of individual females yielded an average ± SD of 21.3 ± 7.5 mg of skin lipids per female. Subsequent fractionation of the lipids yielded an average ± SD methyl ketone fraction of 1.4 ± 1.1 mg/female. Overall, the methyl ketones accounted for an average ± SD of 6.1 ± 3.4% of the skin lipids collected from the female snakes. After accounting for variation in skin surface area, individual females were found to vary widely in the quantity of methyl ketones expressed on their dorsal surface (range = 1.8–16.4 μg/cm^2; Table 2). There was not, however, a significant relationship between the female SVL and the amount of methyl ketones extracted per unit of surface area (Pearson product-moment correlation: $r = 0.169$, $N = 20$, $P = 0.476$).

TABLE 2. QUANTITATIVE AND QUALITATIVE VARIATION IN EXPRESSION OF SEXUAL ATTRACTIVENESS PHEROMONE AMONG INDIVIDUAL FEMALE RED-SIDED GARTER SNAKES, *Thamnophis sirtalis parietalis*[a]

Individual female	Snout-vent length (cm)	Methyl ketone expression ($\mu g/cm^2$)	Unique methyl ketones observed	Relative (%) methyl ketone concentrations	
				Saturated	Unsaturated
1	48.0	5.6	17	75.1	24.9
2	50.4	2.5	12	74.8	25.2
3	54.0	2.2	17	70.3	29.7
4	55.5	2.3	18	58.5	41.5
5	55.6	4.3	18	53.0	47.0
6	56.0	2.1	15	55.9	44.1
7	57.5	16.4	18	49.1	51.9
8	58.5	2.0	16	70.8	29.2
9	59.2	7.0	17	71.5	28.5
10	59.8	1.8	10	17.5	82.5
11	60.4	1.9	15	57.4	42.6
12	61.3	3.4	14	11.1	88.9
13	61.8	3.4	15	60.3	39.7
14	62.0	3.6	18	54.1	45.9
15	66.6	5.4	14	26.3	73.7
16	68.5	5.4	13	13.7	86.3
17	69.1	4.9	10	11.6	88.4
18	69.6	2.5	13	7.4	92.6
19	71.5	10.2	15	20.4	79.6
20	74.2	5.3	16	25.2	74.8

[a] Female snakes are arranged according to snout-vent length (small to large).

Pheromone Quality. Complete GC-MS analysis of the methyl ketone fractions revealed the presence of 18 unique long-chained methyl ketones (Figure 3). Nine of these were identified as long-chain saturated methyl ketones, while the remaining nine had mass spectra in accord with long-chain ω-9 *cis*-unsaturated methyl ketones (Mason et al., 1990). The saturated methyl ketones ranged in size from 394 mass units to 506 mass units, whereas the unsaturated methyl ketones ranged in size from 420 mass units to 532 mass units. Individual females were found to vary in the number of methyl ketones expressed in their skin lipids, ranging from pheromone profiles composed of 10 unique methyl ketones to pheromone profiles composed of all 18 unique methyl ketones (Table 2). However, when we plotted the number of unique methyl ketones present against the SVL of the females, a significant relationship was not observed (Pearson product-moment correlation: $r = -0.338$, $N = 20$, $P = 0.144$).

The relative contribution of the saturated and unsaturated methyl ketones to the overall pheromone profiles varied extensively among females (Table 2).

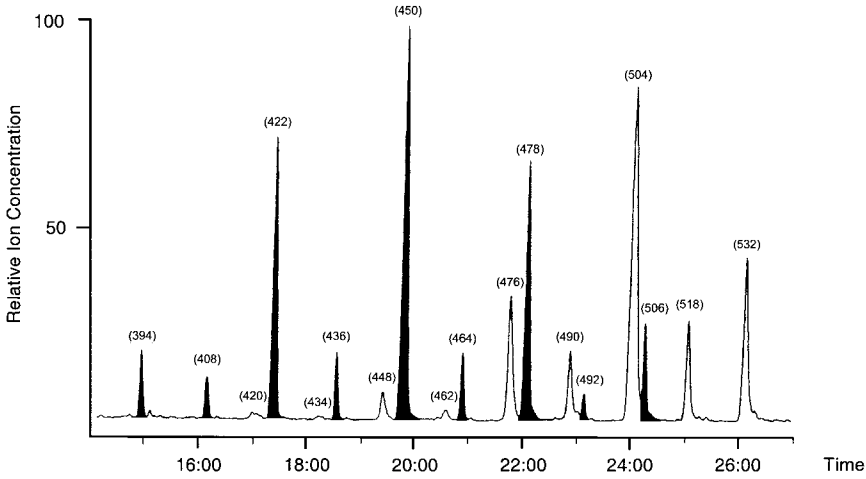


FIG. 3. Gas chromatogram of the female sexual attractiveness pheromone profile for the red-sided garter snake, *Thamnophis sirtalis parietalis*. Pheromone profiles are composed of saturated (shaded peaks) and unsaturated (open peaks) methyl ketones. The numbers over the peaks represent the molecular weights of individual components.

For example, one female expressed a pheromone profile composed of 75.1% saturated methyl ketones and 24.9% unsaturated methyl ketones, whereas a second female expressed a profile composed of 7.4% saturated methyl ketones and 92.6% unsaturated methyl ketones. Saturated methyl ketones composed (average $\pm$ SD) $44.2 \pm 24.5\%$ of the total methyl ketones in the pheromone profiles, and unsaturated methyl ketones composed $55.8 \pm 24.5\%$. When we plotted the SVL of the females against the relative contribution of unsaturated methyl ketones to the pheromone profiles, a significant correlation was observed (Pearson product moment correlation: $r = 0.788$, $N = 20$, $P < 0.001$; Figure 4). This relationship showed that smaller females express pheromone profiles comprised predominantly of saturated methyl ketones, while larger females express profiles composed predominantly of unsaturated methyl ketones.

DISCUSSION

The results of this study demonstrate that adult red-sided garter snakes are able to discriminate among females of various size based solely on chemical cues contained within the skin lipids of the female. Males displayed a courtship preference for large females over small females when presented both simultaneously and in isolation, and continued to demonstrate a similar preference when visual, tactile, and behavioral cues from the females were removed through the use of skin lipid

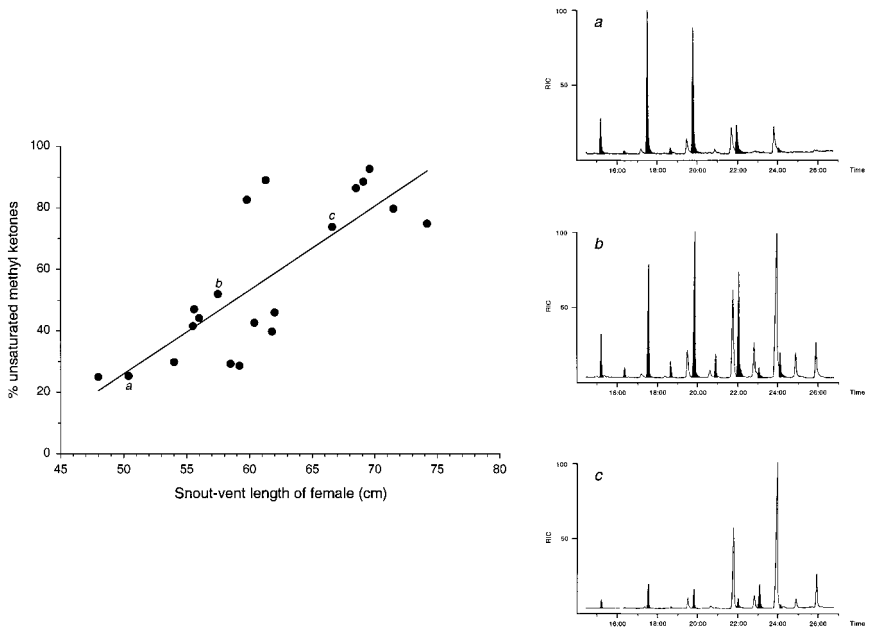


FIG. 4. Regression of the percent relative concentration of unsaturated methyl ketones composing the sexual attractiveness pheromone profile on snout-vent length for female red-sided garter snakes, *Thamnophis sirtalis parietalis*. Small females express pheromone profiles consisting predominantly of saturated methyl ketones (a), mid-size females express profiles consisting of equal proportions of saturated and unsaturated methyl ketones (b), and large females express profiles consisting predominately of unsaturated methyl ketones (c).

extracts. Furthermore, we observed size-specific variation in the chemical structure of the female sexual attractiveness pheromone, suggesting that this pheromone represents the skin lipid-borne cue responsible for mediating male mate choice in this species. Previous studies have documented chemically mediated mate choice in a variety of vertebrates [e.g., mammals: lemmings (Huck and Banks, 1982), rabbits (Reece-Engel, 1988), amphibians: salamanders (Verrell, 1985; Marco et al., 1998), reptiles: lizards (Martín and López, 2000)] but have focused exclusively on the behavioral responses of individuals to chemical cues. To our knowledge, this study represents the first attempt to provide empirical evidence towards the identification of a chemical cue responsible for mediating mate choice in a vertebrate species.

Our results from the behavioral experiments confirm previous studies documenting a courtship preference by male red-sided garter snakes for larger females during the breeding season (Hawley and Aleksiuk, 1976; Garstka et al., 1982; Shine et al., 2001). Similar courtship preference by males for larger females has been observed in other reptiles including lizards (e.g., Olsson 1993; Cooper and Vitt, 1997; Whiting and Batemen, 1999) and snakes (e.g., Luiselli, 1996). One potential benefit incurred by male red-sided garter snakes for choosing a female of larger body size is an increase in the number of offspring sired. Fecundity has been shown to increase with body size in a variety of animals (reviewed in Andersson, 1994), and this appears true for red-sided garter snake populations in Manitoba, where larger females are observed producing larger clutches of young (Gregory, 1977; R. T. Mason, unpublished data).

We are confident that our bioassay results represent a true measure of male courtship preference for skin lipids from larger females. When male red-sided garter snakes were presented with skin lipid extracts from both large and small females, we observed courtship behavior closely mimicking what was observed with the actual females, including chin-rubbing and body alignment (with edge of filter paper). These behaviors are only observed in a reproductive context (Mason, 1993), confirming that we were measuring a courtship response and not some other male behavior (e.g., aggregation). Furthermore, analysis of the treated filter papers showed that similar amounts of skin lipids were transferred to the filter papers from the large and small snakes. Potentially, variation in the total amount of skin lipids transferred for the two female sizes could have driven the male courtship preference observed. Instead, our results suggest that there is some intrinsic property of the female skin lipids that allows for males to differentiate among large and small females.

The observed relationship between the SVL of females and the methyl ketone group ratios demonstrates that the sexual attractiveness pheromone can function as a reliable indicator to males of female body size. Indeed, the observed uniform shift in methyl ketone group ratios as females increased in size conforms well with the observed uniform change in male courtship behavior displayed to females of varying size when tested in the field (Hawley and Aleksiuk, 1976). In addition,

the pattern of variation we observed in pheromone composition is concordant with what would be expected for such variation to function in male mate selection. Although both saturated and unsaturated methyl ketones are required to elicit full male courtship behavior in red-sided garter snakes, unsaturated methyl ketones appear to be the more biologically active. When presented in isolation, unsaturated methyl ketones elicit a five-fold increase in male response over saturated methyl ketones (Mason et al., 1989). Accordingly, higher levels of unsaturated methyl ketones in the pheromone ratios of larger females, as observed in this study, are a likely explanation for why males find these females more attractive.

Why larger females express higher levels of unsaturated methyl ketones is unclear. The production and expression of this pheromone appears to be under hormonal control [estrogen (Crews, 1976; Crews et al., 1984)], suggesting that variation in the release rate of hormones and/or sensitivity of target cells to the hormones may explain the variation. For example, larger females produce a greater number of follicles in the fall [hence, greater numbers of offspring (Gregory, 1977)], which may translate into higher levels of estrogen circulating in the blood during the period of pheromone biosynthesis. Alternatively, females of varying size may differ in the organization of biosynthetic pathways involved in methyl ketone production. Future studies are necessary to resolve the underlying mechanism(s) responsible for the observed variation in methyl ketone production.

We might have expected to find variation in the quantity of sexual attractiveness pheromone expressed by female red-sided garter snakes of varying size or variation in the presence or absence of individual methyl ketones. Similar mechanisms of mate choice have been observed in insects (reviewed in Andersson, 1994). For example, female rattlebox moths (*Utetheisa ornatrix*) prefer to mate with males producing higher quantities of a courtship pheromone (Dussourd et al., 1991), whereas female Oriental moths (*Grapholita molesta*) preferentially court males expressing a food-related chemical in their pheromone blend (Löfstedt et al., 1989). However, we did not observe a correlation between female body size and either of the two measures in the expression of the sexual attractiveness pheromone, suggesting that such variation is not utilized by male red-sided garter snakes to differentiate among females.

Although the results from this study strongly suggest that male red-sided garter snakes differentiate among females of varying size by utilizing size-specific variation in the female sexual attractiveness pheromone, we can not rule out the possibility that additional cues may assist males in mate choice for this species (Shine et al., 2001). For example, male garter snakes searching for sexually attractive females in the field are initially attracted to movement (Joy and Crews, 1988; Holtzman, 2001). Thus, males might initially respond to perceived areas of greater movement (i.e., larger female moving through grass or action of larger mating ball) and then confirm their choice by sampling the skin lipid composition of the female once contact is made. In addition, tactile cues may provide additional

relevant information to the male. When a male initially comes in contact with a female, he proceeds to chin-rub up and down the female, turning back at the head and tail before finally coming to rest with his head resting behind the female's head (Noble, 1937). Males perform this behavior to detect quickly whether the female is mated by means of postcopulatory pheromones deposited near the cloaca (Shine et al., 2000), but males might also be able to judge the size of the female based on distance covered. Further studies are required to determine to what extent these additional cues may be utilized.

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VOLATILE COMPOUNDS IN ANAL GLAND OF SIBERIAN WEASELS (*Mustela sibirica*) AND STEPPE POLECATS (*M. eversmanni*)

JIAN-XU ZHANG,[1] LIXING SUN,[2] ZHI-BIN ZHANG,[1,*]
ZU-WANG WANG,[1] YI CHEN,[1] and RUI WANG[1]

[1]*State Key Laboratory of Integrated Management of Pest Insects and Rodents in Agriculture*
Institute of Zoology, Chinese Academy of Sciences
Beijing, 100080, China

[2]*Department of Biological Sciences*
Central Washington University
Ellensburg, Washington, 98926-7537

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Abstract—The volatile constituents in anal gland secretions of two sympatric *Mustela* species, the Siberian weasel (*M. sibirica*) and steppe polecat (*M. eversmanni*), were studied by the headspace technique, followed by gas chromatography–mass spectrometry (GC-MS) analysis. Nine sulfur-containing compounds were identified. They were 2,2-dimethylthietane, (*Z*)- or (*E*)-2,4-dimethylthietane, (*E*)-2,3-dimethylthietane, 2-ethylthietane, (*E*)-2-ethyl-3-methylthietane, (*Z*)-2-ethyl-3-methylthietane, 2-propylthietane, 3,3-dimethyl-1,2-dithiacyclopentane, and (*Z*)-3,4-dimethyl-2,2-dithiacyclopentane. Among them, (*E*)-2-ethyl-3- methylthietanes, (*Z*)-2-ethyl-3-methylthietanes, and (*Z*)-3,4-dimethyl-1,2-dithiacyclopentane were present in the polecat but not in the weasel. The predominant compound was 2,2-dimethylthietane in the weasel and (*E*)- or (*Z*)-2,4-dimethylthietane in the polecat. These differences were consistent between the two species, regardless of sex and age and, therefore, could possibly be used for species recognition. In the weasel, 2-ethylthietane was found only in the female, and the relative abundance of several compounds was significantly different between males and females. In the polecat, although no sex-specific volatile compounds were found, males and females differed in the relative abundance of several of the compounds. In both species, the relative abundance of some compounds varied with age. We conclude that these volatile compounds can be used to communicate information about species, sex, and age.

Key Words—Anal gland secretion, volatile compounds, Siberian weasel, *Mustela sibirica*, steppe polecat, *Mustela eversmanni*.

* To whom correspondence should be addressed. E-mail: Zhangzb@panda.ioz.ac.cn

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INTRODUCTION

Chemical communication plays a vital role in mammalian social interactions, such as recognition of species, sex, age, dominance status, and physiological and reproductive condition (e.g., Müller-Schwarze, 1974; Brown, 1979; Sun and Müller-Schwarze, 1999). Our current knowledge of animal chemical communication has been mainly garnered through two inseparable approaches: chemical analysis of secretion materials and behavioral bioassays. Chemical analysis of secretions can provide insight into what information is available and how it is coded and, therefore, can narrow or generate new hypotheses for behavioral bioassays (Sun, 1996).

Carnivores, rodents, and ungulates are among the most intensively studied mammalian groups for chemical communication (see Brown and Macdonald, 1985). The chemical composition of the anal gland secretion of small carnivores is of particular interest to researchers. In the *Mustela* genus, chemical analysis has been conducted in the domestic American mink (*M. vison*) (Sokolov et al., 1980; Brinck et al., 1978, 1983), stoat (*M. erminea*) and domestic ferrets (*M. putorius furo*) in New Zealand (Crump, 1980a,b; Crump and Moors, 1985), and mountain weasel (*M. nivalis*) and ferret (*M. putorius*) (Brinck et al., 1983). These efforts have resulted in the identification of 18 major volatile compounds unique to *Mustela*. They are 2-pentylthietane ($C_8H_{16}S$), 3-propyl-1,2-dithiolane ($C_6H_{12}S$), indole (C_8H_7N), and *o*-aminoacetophenone (C_8H_9NO), six isomers of $C_5H_{10}S$, four isomers of $C_6H_{12}S$, and four isomers of $C_5H_{10}S_2$. Among them, two are nitrogen-containing compounds: indole (in all *Mustela* species) and *o*-aminoacetophenone (only in *M. erminea*), and the remaining are all sulfur-containing compounds. These 18 compounds apparently are unique to *Mustela* and have not been found in other genera in the family Mustelidae, such as *Martes martes* (Brinck et al., 1983), *Lutra lutra* (Gorman et al., 1978; Brinck et al., 1983) and *Meles meles* (Brinck et al., 1983). Comparisons of these volatiles from the anal gland secretions of various *Mustela* species show that the presence or absence or relative proportion of isomers of $C_5H_{10}S$, $C_6H_{12}S_2$ and $C_5H_{10}S_2$ is species specific. This provides cues for species recognition among sympatric species.

Findings in intersexual or individual variation in the chemical composition of anal gland secretions may also indicate the possibility for sex and individual recognition within a *Mustela* species. For example, the chromatographic peak profile of the anal gland secretion in the mink is characteristic of the individual and not affected by season (Brinck et al., 1978). In stoats, highly volatile components in the anal gland secretion also demonstrate a stable individual pattern (Erlinge et al., 1982). Sexual dimorphism in the chemical composition of anal gland secretion varies with species. American minks show no specific sex differences over the four seasons (Brinck et al., 1978). Secretions from female stoats during the breeding season, however, contain compounds not present in the male in the breeding season

(Crump, 1980a). This implies that other unidentified compounds may also be involved in sex recognition.

Siberian weasels (*Mustela sibirica fortanieri*) and steppe polecats (*Mustela eversmanni admirata*) distribute sympatrically in farmlands in northern China. They overlap in their activity range and rodent diet (Gao, 1987). Thus, both interspecific and intraspecific recognition is important for the two species. Since secretions from the anal gland have already been identified as a source of information for recognition in *Mustela*, chemical analyses of the volatile compounds may allow us to pinpoint the components involved. In this study, we characterized the volatile components of the two *Mustela* species and explored their potential roles by examining species-, sex-, and age-related differences in these compounds.

METHODS AND MATERIALS

Collection and Separation of Volatile Compounds. Anal glands were removed fresh from Siberian weasels (70 males and 50 females) and steppe polecats (40 males and 20 females) captured legally by fur trappers in several farmlands in central northern China in January 2000. They were immediately frozen and transferred to the laboratory and stored at -20°C until use.

For both species, we used the baculum to estimate the age of males. Animals with a baculum weight <250 mg were classified as juveniles and >350 mg as adults (Sheng and Lu, 1976; Gao, 1987). For females, we used body weight to estimate the age. Those weighing >500 g were classified as adults and <350 g as juveniles (Sheng and Lu, 1976; Gao, 1987). We found no juvenile female steppe polecats and, thus, this age group was not included in our study.

Each anal gland was punctured, and secretion material was squeezed into a 1.5-ml vial. Secretions from individuals of the same species, sex, and age group were pooled into larger vials, each containing samples from at least five individuals. These pooled samples were used for chemical analysis.

For each pooled sample, we filtered 150 mg of secretion material and placed it into a 100-ml glass entrainment jar, which was waterbathed at a constant temperature of $55 \pm 1^{\circ}$C. A headspace sample was collected using Porapak Q (100 ± 2 mg, mesh 80–100). Volatiles trapped on the porous Porapak Q were released by solvent desorption. Porapak Q was packed individually into a glass tube (8 cm long $\times$ 3 mm ID) and kept in place with silanized glass wool plugs. The Porapak Q tube was immersed in ice-water (0°C) for better retention of the volatiles. Volatile chemicals from the headspace of the secretion samples were drawn onto the polymer for 10 hr by using Porapak Q-filtered nitrogen gas at a flow rate of 100 ml/min. At the end of entrainment, trapped volatiles were eluted from Porapak Q with 5 ml of redistilled dichloromethane. The dichloromethane solutions were later condensed to 1/8 volume (0.625 ml) by a gentle stream of N_2 gas and were used for the following analysis.

Gas Chromatography–Mass Spectrometry (GC-MS). Analytical GC-MS was performed on a Shimadzu GC-MS QP5050A instrument. The GC was equipped with a 30-m glass capillary column (0.25 mm ID) coated with SE54. Helium was used as the carrier gas at a flow rate of 0.5 ml/min. The temperature of the injector was set at 250°C, and the split ratio was set at 1:10. The oven temperature was programmed as 50°C for 5 min, then increased at 10°C/min to 220°C, and finally held at 220°C for 10 min. Electron impact was used for ionization, which was set at 70 eV and 250°C.

We searched the literature to determine which reference substances should be used for parallel analyses. This led to our identification of 2,2-dimethylthietane (**1**), 2-ethylthietane (**4**), and 3,3-dimethyl-2,2-dithiacyclopentane (**8**), which had been identified in the mink by using a similar method (Brinck et al., 1978, 1983; Sokolov et al., 1980). (The number that follows each compound corresponds to the number in Table 1 below.) The GC profile and mass spectrum of 2-propylthietane (**7**) was compared with that of a commercially available authentic chemical compound. We used the mass spectra available in the literature to aid in the identification of the following compounds: (*Z*)-or (*E*)-2, 4-dimethylthietane (**2**), (*E*)-2,3-dimethylthietane (**3**) (Crump, 1980b), (*E*)-2-ethyl-3-methylthietanes (**5**), (*Z*)-2-ethyl-3-methylthietanes (**6**) (Crump and Moors, 1985), and (*Z*)-3,4-dimethyl-1, 2-dithiacyclopentane (**9**) (Crump, 1980b; Sokolov et al., 1980; Brinck et al., 1983).

We used the method developed by Sun and Müller-Schwarze (1998) to quantify the relative abundance of the nine compounds. We converted the peak area of a particular compound into the percentage of the summed peak areas of all nine compounds in a sample. We then used a two-tailed independent *t* test to test for the difference in these compounds between species, sexes, and age groups. The level of significance was set at 0.05. Because we had four samples for each specific sex or age group, the degree of freedom was 6 for all tests.

RESULTS

Volatile Compounds in Anal Gland Secretions. Typical GC chromatogram profiles of volatiles from anal gland secretions in male steppe polecats and female Siberian weasels are shown in Figure 1. Mass spectral information of the numbered peaks is given in Table 1. Using headspace sampling and subsequent GC-MS analysis, we identified nine volatiles in the anal gland secretion of the two species: 2,2-dimethylthietane (**1**), (*Z*)-or (*E*)-2,4-dimethylthietane (**2**), (*E*)-2, 3-dimethylthietane (**3**), 2-ethylthietane (**4**), (*E*)-2-ethyl-3-methylthietanes (**5**), (*Z*)-2-ethyl-3-methylthietanes (**6**), 2-propylthietane (**7**), 3,3-dimethyl-1,2-dithiacyclopentane (**8**), and (*Z*)-3,4-dimethyl-1,2-dithiacyclopentane (**9**) (Table 1).

The GC chromatogram shows nine volatile constituents in the secretion of the steppe polecat (Figure 1A). Compounds **1–4** (Table 1) have a molecular mass of

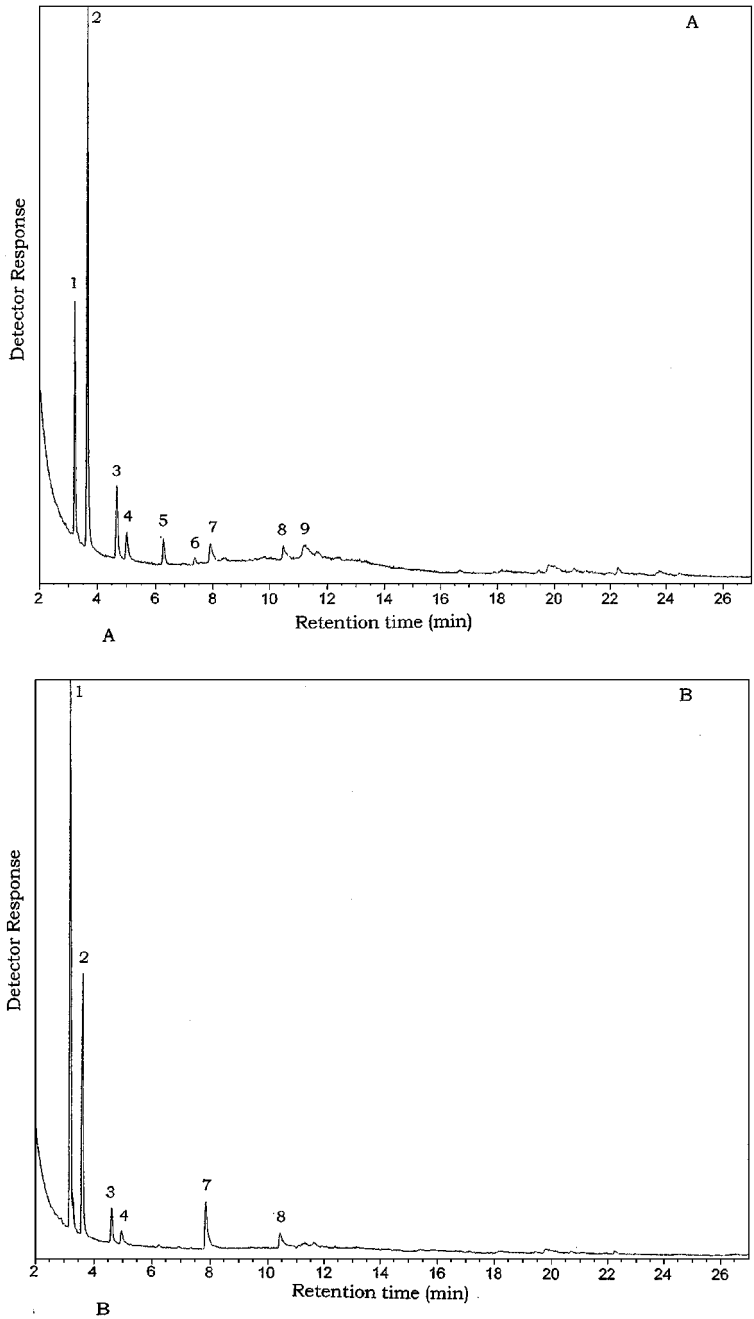


FIG. 1. Gas chromatogram of the anal gland secretion from male adult steppe polecats (*Mustela eversmanni admirata*) (A) and female adult Siberian weasels (*Mustela sibirica fortanieri*) (B).

TABLE 1. MS DATA AND COMPOUNDS IDENTIFIED IN ANAL GLAND VOLATILES OF SIBERIAN WEASEL OR STEPPE POLECAT

Compound	Mass spectrum data [*m*/*z* values (ion intensities)]	Identification
1	102(48), 87(28), 74(28), 69(25), 68(18), 67(15), 60(25), 56(82), 41(100)	2,2-dimethylthietane
2	102(32), 87(3), 74(1), 73(1), 69(4), 68(2), 67(3), 60(100), 61(16), 59(22), 56(31), 45(26), 41(45)	*Z*- or *E*-2,4-dimethylthietane
3	102(30), 87(12), 74(7), 73(7), 69(8), 67(8), 61(15), 60(100), 59(20), 56(28), 41(45)	*E*-2,3-dimethylthietane
4	102(64), 87(42), 74(49), 73(52), 69(14), 68(24), 67(18), 61(13), 60(100), 59(12), 56(29), 55(50), 45(89), 41(100)	2-ethylthietane
5	116(59), 101(22), 87(59), 74(16), 73(42), 69(27), 68(8), 67(51), 60(39), 59(19), 55(70), 45(77), 41(100)	*E*-2-ethyl-3-methylthietanes
6	116(37), 86(6), 84(13), 74(100), 70(19), 69(26), 60(2), 59(27), 56(18), 55(45), 45(29), 41(98)	*Z*-2-ethyl-3-methylthietanes
7	116(55), 88(43), 87(79), 86(26), 74(13), 73(73), 69(45), 67(50), 56(52), 55(51), 41(100)	2-propylthietane
8	134(20), 86(2), 70(10), 69(99), 67(8), 59(14), 57(4), 56(4), 55(13), 53(11), 41(100)	3,3-dimethyl-1,2-dithiacyclopentane
9	134(31), 86(12), 85(13), 70(24), 69(100), 57(42), 55(32), 41(87)	*Z*-3,4-dimethyl-1,2-dithiacyclopentane

102 ($C_5H_{10}S$). Compounds **1** and **4** have GC and MS data identical to peaks identified as 2,2-dimethylthietane and 2-ethylthietane by Brinck et al. (1978, 1983) in a parallel analysis of the anal gland secretion of the mink. Compounds **2** and **3** show mass spectra that are superimposable on those of (*Z*)- or (*E*)-2,4-dimethylthietane and (*E*)-2,3-dimethylthietane found in the domestic ferret (Crump, 1980b) and mountain weasel (Brinck et al., 1983). GC retention time sequence shows that compound **2** is (*Z*)- or (*E*)-2,4-dimethylthietane and compound **3** is (*E*)-2, 3-dimethylthietane. Compounds **5**, **6**, and **7** all have a molecular mass of 116 ($C_6H_{12}S$) (Table 1). Compound **7** and authentic synthetic 2-propylthietane have identical GC and MS data, whereas the MS data of compounds **5** and **6** correspond to (*E*)- and (*Z*)-2-ethyl-3-methylthietane identified in the stoat (Crump and Moors, 1985). The GC and MS data of compounds **8** and **9** show that they both have a molecular mass of 134 ($C_6H_{12}S_2$) (Table 1). The presence of ions at *m*/*z* 41, *m*/*z* 69, and *m*/*z* 134 and the absence of an ion at *m*/*z* 29 indicate that they are most likely isomers of 3,3-dimethyl-1,2-dithiacyclopentane. The GC and MS data of compound **8** are similar to those of 3,3-dimethyl-1,2-dithiacyclopentane (Sokolov et al., 1980). The absence of ions at *m*/*z* 102 and 94 in the MS of compound **9** agrees with that observed by Crump (1980b) for (*Z*)-3, 4-dimethyl-1,2-dithiacyclopentane in the ferret.

Interspecific Differences. In the Siberian weasel, the gas chromatograms and mass spectra of anal gland secretions of both sexes and two age groups had six peaks (Figure 1B) corresponding to 2,2-dimethylthietane (peak 1), (*Z*)- or (*E*)-2,4-dimethylthietane (peak 2), (*E*)-2,3-dimethylthietane (peak 3), 2-ethylthietane (peak 4), 2-propylthietane (peak 7), and 3,3-dimethyl-1,2-dithiacyclopentane (peak 8).

(*E*)-2-Ethyl-3-methylthietanes (peak 5), (*Z*)-2-ethyl-3-methylthietanes (peak 6), and (*Z*)-3,4-dimethyl-1,2-dithiacyclopentane (peak 9) were present in the polecat but not in the weasel. Several compounds, although present in both species, showed a consistent difference in relative abundance between the two species. 2,2-Dimethylthietane was the dominant component in the weasel (male: $t = 5.022$, $P = 0.004$; female: $t = 4.038$, $P = 0.006$), while in the polecat (*Z*)- or (*E*)-2,4-dimethylthietane was the dominant component (male: $t = 4.975$, $P = 0.002$; female: $t = 2.455$, $P = 0.048$). For males, 2-ethylthietane occurred only in the polecat but not in the weasel. The polecat also had a higher level of (*E*)-2,3-dimethythietane ($t = 3.272$, $P = 0.017$) and a lower level of 3,3-dimethy-1,2-dithiacyclopentane ($t = 3.490$, $P = 0.012$) than the weasel. For females, the polecat had a higher level of 2-ethylthietane and a lower level of 2-propylthietane ($t = 3.811$, $P = 0.009$) than the weasel (Table 2).

Intraspecific Differences. In terms of *sex difference*, in Siberian weasels, 2-ethylthietane (peak 4) only occurred in females and was undetectable in males. Furthermore, the relative abundance of (*Z*)- or (*E*)-2,4-dimethylthietane (peak 2) and (*E*)-2,3-dimethylthietane (peak 3) was significantly higher in females than in males ($t = 4.954$, $P = 0.003$ and $t = 2.927$, $P = 0.026$, respectively).

TABLE 2. COMPARISON OF ANAL GLAND VOLATILES BETWEEN SEXES AND CONSPECIES IN SIBERIAN WEASEL AND STEPPE POLECAT[a]

Component	Adult Siberian weasel		Adult steppe polecat	
	Males	Females	Males	Females
1	$78.37 \pm 26.33^{\dagger\dagger}$	$55.68 \pm 21.22^{\dagger\dagger}$	15.99 ± 3.88	14.14 ± 5.09
2	$11.06 \pm 3.67^{\dagger\dagger}$	$26.74 \pm 5.86^{**\dagger}$	47.92 ± 16.75	39.01 ± 15.47
3	$1.97 \pm 0.52^{\dagger}$	$4.41 \pm 0.96^{*}$	7.78 ± 2.07	6.83 ± 3.01
4		$2.13 \pm 0.29^{**\dagger}$	3.92 ± 0.78	4.97 ± 1.59
5			3.54 ± 0.54	$10.07 \pm 2.27^{*}$
6			1.19 ± 0.09	$4.12 \pm 1.04^{*}$
7	7.83 ± 2.31	$8.51 \pm 2.72^{\dagger\dagger}$	5.12 ± 1.69	$2.89 \pm 0.98^{*}$
8	$10.42 \pm 2.89^{\dagger}$	$2.53 \pm 1.02^{*}$	2.44 ± 0.19	$4.15 \pm 0.88^{*}$
9			3.82 ± 1.07	$10.77 \pm 3.00^{*}$

[a] Mean of percent peak area (%) $\pm$ SD, $N = 4$. †:$P < 0.05$; ††:$P < 0.01$ between the two species of the same sex (marked only in the weasel data); *:$P < 0.05$; **:$P < 0.01$ between the two sexes of the same species (marked only in the female data).

Furthermore, 3,3-dimethyl-1,2-dithiacyclopentane (peak 8) was more abundant in males than in females ($t = 3.27$, $P = 0.017$; see Table 2).

In the steppe polecat, five compounds showed quantitative differences between the two sexes. (*E*)- and (*Z*)-2-Ethyl-3-methylthietanes (peaks 5 and 6), 3,3-dimethyl-1,2-dithiacyclopentane (peak 8), and (*Z*)-3,4-dimethyl-1,2-dithiacyclopentane (peak 9) were all higher in females ($t = 2.990$, $P = 0.034$; $t = 2.371$, $P = 0.046$; $t = 2.450$, $P = 0.043$; and $t = 3.318$, $P = 0.012$, respectively), whereas 2-propylthietane (peak 7) was higher in males ($t = 2.762$, $P = 0.038$). However, no sex-specific compound was observed (Table 2).

In terms of *age difference*, in male Siberian weasels, adults had a higher level of 2-propylthietane (peak 7) and 3,3-dimethyl-1, 2-dithiacyclopentane (peak 8) than juveniles ($t = 2.978$, $P = 0.034$ and $t = 3.097$, $P = 0.026$, respectively). In female weasels, adults had a higher level of (*Z*)- or (*E*)-2,4-dimethylthietane (peak 2), (*E*)-2,3-dimethylthietane (peak 3), and 2-propylthietane (peak 7) than juveniles ($t = 2.608$, $P = 0.041$; $t = 2.990$, $P = 0.032$ and $t = 3.582$, $P = 0.014$, respectively). Juveniles, however, had a higher level of 3,3-dimethyl-1, 2-dithiacyclo pentane (peak 8) than adults ($t = 3.049$, $P = 0.026$).

In male steppe polecats, adults had a lower level of 2-ethylthietane (peak 4) and (*E*)-2-ethyl-3-methylthietanes (peak 5) in comparison with those in juveniles ($t = 2.976$, $P = 0.383$ and $t = 2.901$, $P = 0.0371$, respectively; Table 3).

DISCUSSION

Our results show that all volatiles detected in the anal gland secretion of the Siberian weasel and steppe polecat were sulfur-containing compounds, as previously reported in other *Mustela* species (Sokolov et al., 1980; Crump, 1980a,b; Brinck et al., 1983; Crump and Moors, 1985). A unique aspect that has not been previously reported, however, is that four isomers of $C_5H_{10}S$ occurred in either Siberian weasels or steppe polecats. Whether this similarity in the anal gland secretions is due to their close phylogenetic relationship or to the similarity in microbial communities is yet unknown.

Three compounds [two isomers of 2-propylthietane and (*Z*)-dimethyl-1,2-dithiacyclopentane] occurred only in steppe polecats. 2,2-Dimethylthietane was dominant in the Siberian weasel, while (*Z*)- or (*E*)-2,4-dimethylthietane was most abundant in the steppe polecat. The two species also differed significantly in the relative abundance of 2-ethylthietane, (*E*)-2,3-dimenthythietane, 3,3-dimethy-1,2-dithiacyclopentane, and 2-propylthietane for males or females or both. These consistent qualitative and quantitative differences between the two sympatric species provide unambiguous recognition cues between them.

Nitrogen-containing indole, detected previously in other *Mustela* species, was not found in the two species we studied. This can probably be attributed to the headspace sampling technique: our sampling apparatus may fail to trap indole,

TABLE 3. COMPARISON OF ANAL GLAND VOLATILES BETWEEN AGE GROUPS OF SIBERIAN WEASEL AND STEPPE POLECAT[a]

	Male Siberian weasel		Female Siberian weasel		Male steppe polecat	
Compound	Juveniles	Adults	Juveniles	Adults	Juvenile	Adult
1	74.25 ± 30.01	78.37 ± 26.33	64.7 ± 19.88	55.68 ± 21.22	19.38 ± 4.19	15.99 ± 3.88
2	9.90 ± 2.87	11.06 ± 3.67	16.29 ± 7.77	26.74 ± 5.86*	38.39 ± 9.88	47.92 ± 16.75
3	1.82 ± 1.03	1.97 ± 0.52	2.42 ± 0.49	4.41 ± 0.96*	6.87 ± 3.01	7.78 ± 2.07
4			2.52 ± 0.72	2.13 ± 0.29	8.94 ± 3.11	3.92 ± 0.78*
5					6.33 ± 1.83	3.54 ± 0.54*
6					1.53 ± 0.67	1.19 ± 0.09
7	3.07 ± 1.35	7.83 ± 2.31*	3.53 ± 0.81	8.51 ± 2.72*	3.72 ± 0.68	5.12 ± 1.69
8	5.44 ± 1.46	10.42 ± 2.89*	6.82 ± 1.08	2.53 ± 1.02*	3.86 ± 0.94	2.44 ± 0.19
9					5.01 ± 1.82	3.82 ± 1.07

[a] Mean of percent peak area (%) ± SD, $N = 4$. *: $P < 0.05$.

which is less volatile than the nine compounds we identified. In fact, we only found three sulfur-containing compounds (2,2-dimethylthietane, 2-ethylthietane, and 3,3-dimethyl-1,2-dithiacyclopentane) in the anal gland secretion of the mink using our sampling method (unpublished data). In contrast, Brinck et al. (1983) detected indole, in addition to these three compounds in solvent extracts from mink secretions. Thus, the headspace technique appears to be insensitive for detecting relatively heavy volatiles.

Intraspecific communication is suggested to be the main function of the anal gland secretion in mustelids (Erlinge et al., 1982; Brinck et al., 1983). Our study demonstrates that there is a wealth of information contained in the anal gland secretion that can be used for age and sex recognition in the two *Mustela* species. It seems that the coding for sex information takes two forms, either by the presence or absence of sex-specific compounds, such as 2-ethylthietane in female Siberian weasels, and/or by the difference in relative abundance of some compounds between males and females. We failed to find any age-specific compounds.

The sex or age difference in the composition of secretions is generally regulated by hormones (Ebling, 1977). For example, female-specific compounds are only found in stoats during the breeding season (Crump et al., 1980a). In ferrets, production of anal gland secretion is suppressed during estrus (Crump et al., 1980b). Quinaldine is only found in the urine of male red foxes, *Vulpes vulpes* (Jorgenson et al., 1978), and sex hormones significantly affect some volatile constituents in the anal gland secretion of the wolf, *Canis lupus* (Raymer et al., 1985). The hormonal difference between males and females or between adults and juveniles may be responsible for the qualitative and quantitative differences in the volatile compounds in our study.

One obvious caveat is that not all volatiles in anal gland secretions were detected due to methodological limitations (e.g., indole). Additionally, nonvolatiles may also play a significant role (e.g., Sun and Müller-Schwarze, 1998). Whether these nine volatiles are exclusively involved in these recognitions should be ultimately confirmed through behavioral bioassay in the future.

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ANNOUNCEMENT

19TH ANNUAL MEETING OF THE INTERNATIONAL SOCIETY OF CHEMICAL ECOLOGY AUGUST 3–8, 2002, HAMBURG

The 19th annual ISCE meeting will be held at the Department of Chemistry, University of Hamburg, Germany. The conference will begin on the evening of August 3, 2002 (Saturday) and end on the evening of August 7 (Wednesday). The meetings will be hosted by Wittko Francke.

There will be four symposia covering aspects of chemical ecology in symbiosis, molecular biology, insect-plant relationship, and pheromone chemistry.

Both oral and poster presentations are invited from participants. Contributed papers fitting to the symposia are particularly welcome.

Participants are encouraged to arrive on Saturday, August 3, and attend the Social Lecture given by Prof. Dr. Jerrold Meinwald followed by a mixer that evening. The concluding banquet will be held Wednesday evening, August 7.

Further Information may be obtained on the meetings web site http://isce2002.chemie.uni-hamburg.de

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Mini-Review

CONSTRAINTS ON EFFECTIVENESS OF CYANOGENIC GLYCOSIDES IN HERBIVORE DEFENSE

ROSLYN M. GLEADOW* and IAN E. WOODROW

School of Botany, The University of Melbourne
Victoria, Australia 3010

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Abstract—Cyanogenesis is the process by which hydrogen cyanide is released from endogenous cyanide containing compounds. Many cyanogenic plants release HCN in sufficient quantities to be toxic and, as a result, tend to be avoided by herbivores. However, there are many exceptions with some herbivores either immune to the cyanogenic status of the plant, or in some cases attracted to cyanogenic plants. This has led to a certain degree of scepticism regarding the role of cyanogenic glycosides as defense compounds. In this review, we examine evidence showing that differences in the effectiveness of cyanogenic glycosides in deterring herbivory can usually be reconciled when the morphology, physiology, and behavior of the animals, together with the concentration of cyanogenic glycosides in the host plant, are taken into account. Cyanogenic glycosides are not effective against all herbivores, and not all cyanogenic plants release enough cyanide to be considered toxic. Nevertheless, they do form part of the broad spectrum of toxic and distasteful compounds that herbivores must accommodate if they are to feed on cyanogenic plants.

Key Words—Cyanide, herbivory, defense, toxic compounds, diet selection, plant secondary metabolites, cyanogenesis.

INTRODUCTION

Over 2500 plant species have the capacity to release hydrogen cyanide from endogenous cyanide-containing compounds (Conn, 1981; Jones, 1988; Seigler, 1998). Many of these cyanogenic plants are capable of releasing HCN in sufficient

* To whom correspondence should be addressed. E-mail: r.gleadow@unimelb.edu.au

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quantities to be toxic and, as a result, are often avoided by herbivores (Nahrstedt, 1985; Jones, 1998). Nevertheless, there has been a certain amount of controversy over the effectiveness of cyanogenesis as a herbivore defense system (e.g., Hruska, 1988), which has become all the more important to resolve with the recent "engineering" of cyanogenesis into the otherwise acyanogenic *Arabidopsis thaliana* (Tattersall et al., 2001). In this review, we examine studies of cyanogenesis in relation to herbivory and argue that conflicting data can generally be reconciled when the morphology, physiology, and behavior of the herbivores, together with the concentration of cyanogenic glycosides in the host plant, are taken into account.

CYANOGENESIS

The most common cyanogens found in plants are glycosides of α-hydroxynitriles (i.e., cyanohydrins) (Figure 1) (Møller and Poulton, 1993; Lechtenberg and Nahrstedt, 1999; Møller and Seigler, 1999). In cyanogenesis, the sugar moiety is cleaved from the cyanogenic glycoside in a process catalyzed by one or more β-glucosidases (Figure 2). The resulting cyanohydrin is relatively unstable and degrades, either spontaneously or enzymatically, to produce HCN and an aldehyde or a ketone (Conn, 1981). Autotoxicity is prevented by spatial separation—either at the subcellular or tissue level—of the degradative enzymes and the cyanogenic glycoside (e.g., Saunders et al., 1977; Frehner and Conn, 1987; Poulton, 1988; Swain et al., 1992). Tissue disruption, as might happen when tissue is chewed by a herbivore, is necessary for HCN to be released (Nahrstedt, 1985).

Cyanogenic compounds also function in the metabolism and transport of nitrogen (Lieberei et al., 1985; Selmar et al., 1988; Selmar, 1993; Gleadow et al., 1998) and the control of germination (e.g., Esashi et al., 1996). These roles do not, however, need to be seen as an alternative to their role in herbivore defense. Just as some plants store nitrogen as inactive Rubisco (Woodrow, 1994), cyanogenic plants may store nitrogen in a toxic form for the dual purpose of defense.

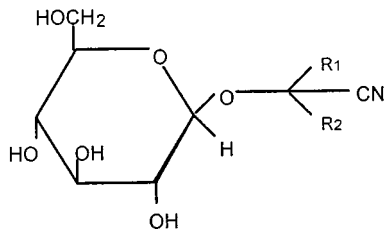


FIG. 1. General structure of cyanogenic glycosides. The aglycone is typically derived from one of the hydrophobic protein amino acids: tyrosine, phenylalanine, valine, leucine, or isoleucine. The glycoside is usually a monoglycoside, although there are a number of diglycosides and one triglycoside (Lechtenberg and Nahrstedt, 1999).

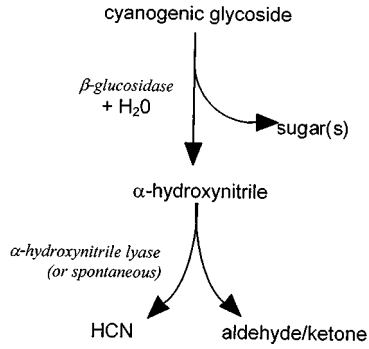


FIG. 2. Degradation of cyanogenic glycosides is a two-step process. First, the molecule is cleaved, resulting in the production of a sugar and a cyanohydrin. The cyanohydrin is unstable and is further degraded, either spontaneously or in the presence of an α-hydroxynitrile lyase, to produce hydrogen cyanide and a carbonyl group (Poulton, 1988).

ASSOCIATION BETWEEN CYANOGENIC PLANTS AND POISONING

The association between certain plants and acute poisoning in humans and domestic animals is well known. There are many well-documented examples of deaths and serious illness from consumption of cyanogenic plants in humans (e.g., Cock, 1982; Cardoso et al., 1998; Banea-Mayambu et al., 2000), cattle (e.g., Robinson, 1930; Finnemore et al., 1935; Boyd et al., 1938; Hopkins, 1995; Zentek, 1997), sheep (e.g., Robinson, 1930; Cooper-Driver et al., 1977; Crush and Caradus, 1995), koalas (e.g., Pratt, 1937), goats (e.g., Webber et al., 1985), and other grazing mammals (e.g., Harborne, 1982; Saucy et al., 1999). Moreover, grazing mammals do not usually eat large quantities of cyanogenic food when alternatives are available (e.g., Jones, 1962; Cooper-Driver et al., 1977; Jones, 1988; Crush and Caradus, 1995; Caradus and Forde, 1996). Many insects and mollusks also tend to avoid cyanogenic plants (e.g., Cooper-Driver and Swain, 1976; Keymer and Ellis, 1978; Dritschilo et al., 1979; Dirzo and Harper, 1982; Foulds, 1982; Schreiner et al., 1984; Nahrstedt, 1985, 1988; Jones, 1988; Kakes, 1989; Patton et al., 1997; Schappert and Shore, 1999b,c; Gleadow and Woodrow, 2000a,b; Magalhaes et al., 2000).

Many of the examples involve studies that are not necessarily conclusive (see, for example, the discussion of Dritschilo et al., 1979, in Hruska, 1988). Nevertheless, there are many cases where it is quite reasonable to attribute feeding patterns to the cyanogenic glycoside content of the plant. Patton et al. (1997), for example, fed leaves from a range of species of *Prunus* to Japanese beetles (*Popillia japonica*) in a fully replicated, no-choice experiment. They found a strong negative correlation between the concentration of the cyanogenic glycoside prunasin in the

leaves and the feeding intensity of the beetles. Moreover, in a parallel study, they incorporated the cyanogenic diglycoside, amygdalin, into artificial diets and found a similar relationship.

Despite evidence documenting the deterrent effect of cyanogenic glycosides, there are a number of studies in which glycosides are shown to have little or no effect on herbivores (e.g., Scriber, 1978; Ferriera et al., 1997; Glander et al., 1989). Indeed, in some cases, plant cyanide production actually acts as a phagostimulant rather than an inhibitor (e.g., Calatayud et al., 1994; Calatayud and Le Rü, 1996; Mowat and Clawson, 1996).

Nearly all of the variability in the effectiveness of cyanogenic glycosides in defense can be explained by four confounding factors. First, the concentration of the cyanogenic glycosides may be below the threshold toxicity. Second, the animal feeding on the species under examination may be a specialist that has evolved mechanisms to cope with high levels of HCN in the diet. Third, the cyanogenic plant might normally be consumed as part of a mixed diet and, therefore, might not be toxic. Fourth, the mode of feeding may be such that the animal does minimal damage to the leaf, thereby limiting the mixing of the cyanogenic glycoside with the degradative β-glucosidases.

CONCENTRATION EFFECTS

Many studies of the effectiveness of cyanogenic compounds in herbivore defense only tested for the presence or absence of cyanogenic glycosides. Natural populations of plants, however, vary widely in cyanogenic glycoside concentration (e.g., Nass, 1972; Hughes, 1991; Thomsen and Brimer, 1997; Schappert and Shore, 1999a,b; Buhrmester et al., 2000; Gleadow and Woodrow, 2000b, Goodger et al., 2002), which in turn, impacts on the degree of herbivory (Feeny, 1976). Gleadow and Woodrow (2000a), for example, detected a significant inverse correlation between the concentration of cyanogenic glycosides in the young leaves of *Eucalyptus cladocalyx* and the amount of damage by herbivores. Similarly, Schappert and Shore (1999b) found that the concentration of cyanogenic glycosides was important not only in the selection of *Turnera ulmifolia* as a source of food, but also as an oviposition site for *Euptoieta hegesia*.

Cyanogenic glycoside concentration also varies with leaf age and environmental conditions (e.g., Patel and Wright, 1958; Bernays et al., 1977; Caradus et al., 1990; Gleadow et al., 1998; Gleadow and Woodrow, 2000a). A particular plant may be innocuous under one set of conditions, but lethal under another (see Bernays et al., 1977). Studies that show little or no influence of cyanogenic glycosides on feeding preferences could be testing plants that are essentially nontoxic to the herbivore. This has been particularly well documented in *Sorghum*, which

is highly toxic to grazing stock when young, but becomes suitable for pasture as plants mature (Martin et al., 1938; McBee and Miller, 1980; Haskins et al., 1987).

SPECIALISTS VERSUS GENERALISTS

Cyanogenesis appears to be an effective inhibitor of feeding by infrequent or opportunistic herbivores rather than by specialists (Compton and Jones, 1985; Nahrstedt, 1985; Hughes, 1992; Schappert and Shore, 1999c, 2000). For example, the feeding and reproductive behavior of the specialist clover weevil (*Sitona lepidus*) is the same on both cyanogenic and acyanogenic *Trifolium repens* (Mowat and Clawson, 1996). *Phenacoccue manihot*, which feeds on *Manihot esculenta*, actually uses the presence of linamarin as a phagostimulant (Calatayud and Le Rü, 1996). By contrast, the generalist-feeding insects *Schistocerca gregaria* and *Sodptera littoralis* avoid feeding on cyanogenic plants (Cooper-Driver and Swain, 1976; Schwarz et al., 1996).

Schappert and Shore (1999c) studied the abundance and diversity of herbivores on populations of *Turnera ulmifolia* in detail on Jamaica. They found that the number of herbivore taxa in any particular population was inversely proportional to the cyanogenic glycoside content of the leaves. However, the amount of herbivory was similar in all populations (1–9% leaf area lost), and most of the damage to cyanogenic plants came from a relatively small suite of insects. Thus, in *T. ulmifolia*, cyanogenesis appears to deter generalist herbivores, but not specialists (Schappert and Shore, 1999c).

Specialist herbivores appear to have evolved mechanisms to tolerate cyanogenic glycosides (e.g., Feeny, 1976; Provenza et al., 1992). Some even use them as a source of nitrogen, as do some fungi (e.g., Birk et al., 1996; Osbourne, 1996). Larvae of the neotropical butterfly, *Heliconius sara*, do this by sequestering the cyanogens from the leaves of the host plant (*Passiflora auriculata*), preventing the release of cyanide. The cyanogen is then metabolized within the insect, and the nitrogen is recovered and used in protein synthesis (Engler et al., 2000). Other specialist herbivores retain the sequestered cyanogenic glycosides and employ them in defense against predators (Nahrstedt, 1985, 1988; Hughes, 1991; Engler et al., 2000). For example, larvae of *Eupitoiea hegesia* take up cyanogenic glycosides from their host plant (*Turnera ulmifolia*) and become distasteful to common predators such as *Anolis* lizards (Schappert and Shore, 1999b). A more complex story is seen in *Lotus corniculatus* (Nahrstedt, 1988). While some snails (e.g., *Helix aspersa*) are deterred from feeding on cyanogenic *L. corniculatus*, larvae of *Zygaena trifolii* are not. These larvae both synthesize and sequester cyanogenic glycosides from *L. corniculatus*, increasing their resistance to predators, similar to *Eupitoiea hegesia*. However, a specialist predator on *Z. trifolii*, *Apanteles*

zygaenarum, is itself able to tolerate HCN, circumventing the complex defense mechanism employed by the *Z. trifolii* larvae (Nahrstedt, 1988).

Specialist herbivores gain a competitive advantage by feeding on plants that are unpalatable to other animals (Edwards, 1989; Bryant et al., 1992). This is well illustrated by the feeding preferences of three sympatric species of bamboo lemur in Madagascar (Glander et al., 1989). The golden bamboo lemur (*Hapalemur aurens*) feeds heavily on the cyanogenic shoots of *Cephalostachym* cv. *viguieri*, consuming up to 12 times the calculated lethal dose of HCN per day. The distribution of the golden bamboo lemur overlaps with that of two other bamboo lemurs (*H. griseus* and *H. sium*), which preferentially feed on the acyanogenic *C. perrieri* and acyanogenic parts of *C. viguieri* (Glander et al., 1989). While the mechanism by which the golden bamboo lemur tolerates such high concentrations remains unknown, it is clear that food preferences have contributed to the sympatric distribution of these primate species.

Most omnivorous mammals are generalists and, in general, are susceptible to cyanide poisoning. The dose required to cause acute poisoning varies with body size and species. The rate of ingestion is also important, as animals can detoxify the cyanide over time (Cock, 1982). Humans are generalists and can only tolerate about 1.4 mg HCN/kg body weight, or about 50–200 mg/day (Harborne, 1982), compared with sheep, which can tolerate about 2.5 mg/kg (Jones, 1972). Unlike other generalists, humans consume many highly cyanogenic plants—two thirds of all crop plants have at least some cyanogenic parts (Jones, 1998)—but because foods are processed before consumption, we do not consume large quantities of the cyanogenic glycosides themselves. Processed food has the added benefit of becoming sweeter through the production of glucose (Jones, 1998; Cardoso et al., 1998).

DIET SELECTION

In practice, animals are unlikely to overingest toxins if they are given a choice in their diet (Provenza et al., 1992). Cyanogenic plants frequently have a bitter taste that is known to deter herbivores (Nahrstedt, 1985). Humans, too, can detect the bitter taste of intact cyanogenic glycosides such as linamarin and lotaustralin, (King and Bradbury, 1995; Jones, 1998). Farmers in Malawi use the degree of bitterness as an indicator of the degree of toxicity of *Manihot esculenta* (Chiwona-Karltun et al., 1998, 2000), although the flavor may be modified by the presence of various organic acids (King and Bradbury, 1995).

Hungry animals are generally less discriminating in their choice of food (e.g., Bernays et al., 1977; Crush and Caradus, 1995; Caradus and Forde, 1996). Webber et al. (1985), for example, documented a case where a herd of 50 goats in western Victoria, Australia, was exposed to large amounts of *Eucalyptus cladocalyx* leaves when a tree was felled. With little alternative pasture due to a drought, the goats

fed ravenously on the young leaves resulting in the death of over half the herd, even after veterinary treatment (Webber et al., 1985).

Results of no-choice feeding experiments may, therefore, seriously underestimate the degree of avoidance that occurs in the field. Young nymphs of *Zonocerus variegatus*, for example, will eat *Manihot esculenta* leaves in captivity when there is no alternative, but do not feed on them at all in the field (Bernays et al., 1977). Even the older, more cyanide-tolerant instars of *Z. variegatus* only feed extensively on *M. esculenta* when they are deprived of other food, resulting in reduced growth rates (Bernays et al., 1977). In binary choice experiments, animals usually choose to eat the acyanogenic plants, thereby avoiding cyanide poisoning. For example, when voles (*Arvicola terrestri*) were offered a choice between cyanogenic and acyanogenic *Trifolium repens*, they consistently preferred the acyanogenic morph. When they were offered only cyanogenic plants (no-choice), they consumed them, but their overall food intake was reduced (Saucy et al., 1999; Viette et al., 2000).

History of exposure to cyanogenic glycosides can also affect feeding patterns (Jones, 1988; Viette et al., 2000). Increased tolerance to amygdalin develops over time in the insect *Spodoptera frugiperda* and is related to improved detoxification of the aglycone (Ferreira et al., 1997). By contrast, habituation to the cyanogenic glycosides amygdalin and salicin by another insect, *Diatraea saccaralia*, has been attributed to the decreased synthesis of endogenous β-glucosidases in the midgut (Ferreira et al., 1997). Similarly, in a controlled experiment, Keymer and Ellis (1978) observed that the mollusks *Helicella itala* and *Cochlicella acuta* ate more leaves on their second exposure to cyanogenic *Lotus corniculatus* than in the first phase of the experiment. The mechanism for this is not clear, particularly as mollusks are known to produce a range of endogenous cyanogenic β-glucosidases (Freeland and Janzen, 1974). Nevertheless, it does illustrate a degree of habituation by the herbivore.

In most animals, gaseous HCN combines with thiosulfate to form less toxic thiocyanate in a process catalyzed by rhodanese (thiosulfate cyanide sulfur transferase, EC 2.8.1.1; Figure 3). Humans and other mammals that regularly consume highly cyanogenic foods increase the production of the rhodanese, and can, therefore, tolerate higher concentrations of cyanide (McMahon et al., 1995). The amount of protein in the diet affects this degree of tolerance. This is particularly true for proteins high in cysteine, as they provide the sulfur essential for the thiosulfate production (Conn, 1991). Problems do arise, however, in humans from long-term ingestion of HCN including hyperthyroidism, goiter, cretinism, tropical ataxic neuropathy, konzo, and tropical diabetes (Cock, 1982; McMahon et al., 1995; King and Bradbury, 1995; Banea-Mayambu et al., 1997). Similar disorders have also been noted in sheep grazing on highly cyanogenic *Trifolium repens* in New Zealand (Crush and Caradus, 1995). As a result of diet selection, food processing and detoxification, acute poisoning from food in humans is rare, even in Africa where consumption rates of *Manihot esculenta* are high (Cock, 1982).

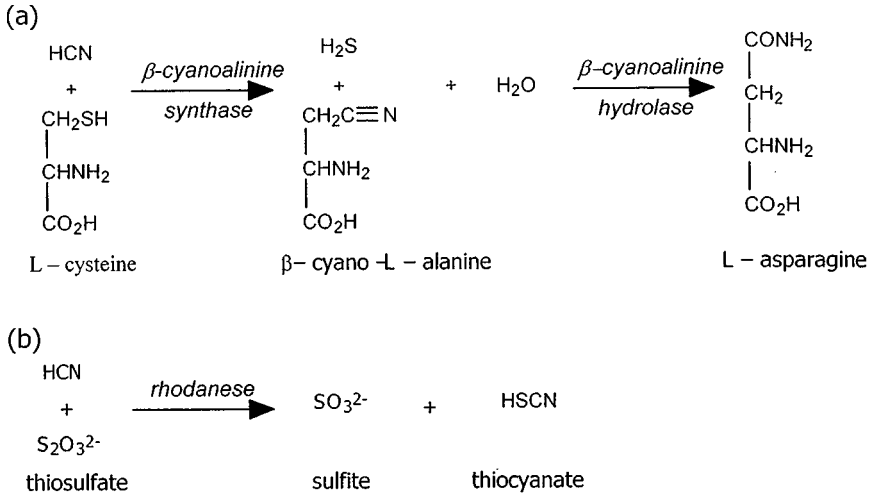


FIG. 3. Detoxification of cyanide by (a) β-cyanoalanine synthase and (b) rhodanese. The former is common in plant tissue and also occurs in some insects. The latter pathway is most commonly found in mammals and microoganisms, but it is also found, to a smaller extent, in plant tissues (Conn, 1981; Manning, 1988).

MODE OF FEEDING

In natural systems, cyanogenesis only occurs when a cyanogenic glycoside is mixed with the appropriate degradative enzymes, for example, when the tissue is chewed. Mammals tend to consume whole leaves (Cork, 1996) and almost inevitably consume β-glucosidases along with the cyanogen. Different orders of insects, however, have different types of mouthparts, which affect their feeding style. Generally, they have either sucking mouthparts (e.g., Hemiptera, Homoptera) or chewing mouth parts (e.g., Coleoptera, Hymenoptera, Orthoptera). If the insect herbivore has sucking mouthparts, then there is minimal tissue disruption, thereby avoiding the defense process. On the other hand, insects that chew leaves tend to mix the cyanogenic glycoside with the degradative enzymes, which are separated at either the cellular or tissue level in the plant (Frehner and Conn, 1987; Poulton, 1988). This difference in mouthparts could explain the difference between, for example, aphids (*Macrosiphum euphorbiae*, Hemiptera) and sawflies (*Strongylogaster impressata*, Hymenoptera), which show neutral and discriminatory feeding behavior, respectively, on polymorphic *Pteridium aquilinum* (Schreiner et al., 1984). One notable exception to this hypothesis is the field study by Dritschilo et al. (1979), who found that aphids fed preferentially on acyanogenic *Trifolium repens*.

The length of the stylus of sucking insects may also determine feeding patterns. Fully mature cassava burrowing bugs (*Cyrtomenus bergi*, Hemiptera) are able to feed effectively by penetrating beyond the outer layers of the root, which are particularly high in linamarin, while the young insects, bearing a shorter stylus, are deterred from feeding (McMahon et al., 1995).

CONCLUSION

The effectiveness of cyanogenesis as an herbivore deterrent is not universal, and this has led to some degree of scepticism. However, variation in the ability to deter herbivores can usually be explained in terms of the concentration of the cyanogenic glycosides themselves, the availability of alternative foods, whether or not the herbivore is a specialist, and the mode of feeding. Cyanogenic glycosides are not effective against all herbivores, and not all cyanogenic plants release enough cyanide to be considered toxic. Nevertheless, they do form part of the broad spectrum of toxic and distasteful compounds that herbivores must overcome if they are to feed on cyanogenic plants.

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(*R*)-(+)-PALASONIN, A CANTHARIDIN-RELATED PLANT TOXIN, ALSO OCCURS IN INSECT HEMOLYMPH AND TISSUES

OLIVER FIETZ,[1] KONRAD DETTNER,[2] HELMAR GÖRLS,[3] KERSTIN KLEMM,[2] and WILHELM BOLAND[1,*]

[1]*Max Planck Institute for Chemical Ecology*
Winzerlaer Str. 10
D-07745 Jena, Germany

[2]*Institute for Animal Ecology II*
University of Bayreuth
D-95440 Bayreuth, Germany

[3]*Institute of Inorganic and Analytical Chemistry*
Friedrich-Schiller University Jena
August-Bebel-Strasse 2, D-07743 Jena, Germany

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Abstract—Gas chromatographic and mass spectroscopic analyses of extracts of cantharidin-containing meloid, clerid, and staphylinid beetles revealed the presence of minor to significant amounts of palasonin, previously only known from seeds and fruits of the Indian shrub *Butea frondosa* (Leguminaceae). Unlike (*S*)-(−)-palasonin (>99% ee) from *B. frondosa*, the insects produce palasonin of low ee with the (*R*)-(+)-enantiomer (0–50% ee) prevailing. The ee of palasonin from individual specimens of predatory insects (*Trichodes apiarius*), which acquire their chemical protection from cantharidin-producing insects, may vary considerably. The absolute configuration of (*S*)-(−)-palasonin, previously deduced from indirect chemical and spectroscopic methods, was confirmed by X-ray crystal structure analysis of a cyclic imide derived from (*S*)-(−)-palasonin and (*S*)-(−)-1-(4-nitrophenyl)-ethylamine.

Key Words—Cantharidin, palasonin, *Butea frondosa*, Meloidae, Cleridae, Staphylinidae, canthariphilous insects.

INTRODUCTION

The terpenoid cantharidin represents one of the most famous defensive compounds from insects. Blister beetles (Meloidae) and false blister beetles (Oedemeridae) are

* To whom correspondence should be addressed. E-mail: Boland@ice.mpg.de

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the only insect species that are able to synthesize this unusual vesicant, insecticide, antifeedant (Carrel and Eisner, 1974; Carrel et al., 1986), and powerful inhibitor of protein phosphatase 2A (PP2A) (Matsuzawa et al., 1987). Since the dynamics of protein phosphorylation and dephosphorylation by protein kinases and protein phosphatases control metabolic activities, cantharidin affects one of the most important regulatory elements of cellular signal transduction (Cohen, 1989). Besides the meloids and oedemerids, other insect orders, collectively called cantharidphilous insects, utilize cantharidin for protection (Dettner, 1997). These insects are not able to produce cantharidin by themselves but are attracted over distances to sources of the toxin and become protected after uptake of the compound from the hemolymph of living or dead meloids, oedemerids, or other cantharidin sources (Frenzel et al., 1992; Dettner, 1997; Hemp and Dettner, 2001). In several species, male-produced cantharidin is transferred during copulation to the female, for incorporation into the eggs (Holz et al., 1994; Eisner et al., 1996).

A structurally related toxin, namely (−)-palasonin, which lacks one of the two angular methyl groups of cantharidin, was isolated in 1967 from seeds of the Indian shrub *Butea frondosa* Koenig ex Roxb. (Leguminaceae), also known as "flame of the forest" due to its brightly orange colored flowers (Raj and Kurup, 1967). Palasonin was found to be effective against ascarids, stomach worms of sheep, oxyurids of mice, and hook worms of dogs (Mehtar and Parasar, 1966). The biochemical mechanism of the antihelminthic action of palasonin is based upon inhibition of glucose uptake affecting the energy generating mechanisms and motor activity of the parasite (Kumar et al., 1995). Like cantharidin, palasonin also inhibits PPA2, albeit with much lower efficiency (Liu et al., 1995).

Although both compounds share the same unique, angularly methylated oxabicyclo skeleton, the toxins have never been reported to cooccur in either plants or insects. However, recent analyses of species from the families Meloidae (Dettner et al., 2001), Cleridae, and Staphylinidae gave the first evidence for simultaneous presence of cantharidin and palasonin in the hemolymph and tissues of individual insects. Since palasonin is a chiral compound, we not only thoroughly documented the occurrence of the two toxins in cantharidin-producing and cantharidin-utilizing insects, but also investigated the absolute configuration and enantiomeric excess (ee) of the compound.

METHODS AND MATERIALS

Collection of Insects

Specimens of Hycleaus lunata Pall. (Meloidae) were caught in 1996 by Prof. Dr. G. Gäde (Rondebosch, South Africa) and transported to Bayreuth by Prof. Dr. E. Zebe (Münster), where they were frozen. Specimens of *Trichodes apiarius* (L.) (Cleridae) were caught from yellow colored composite blossoms at

Saint Jean du Gard (Southern France, Département Gard) on May 30, 1993 by K. Dettner. Rove beetles of *Eusphalerum minutus* (F.) (Staphylinidae) were collected from *Ranunculus* blossoms by K. Dettner near Bayreuth-Seulbitz (Northern Bavaria, Germany) on May 10, 1996.

Extraction of Beetles

Insects were extracted following a standard protocol. Prior to extraction, dry body weight was determined for all samples. Whole specimens, reproductive organs of beetles, eggs, or larvae were treated in small test tubes with 50–300 μl 6N hydrochloric acid (HCl) at 120°C for 4 hr in order to dissolve all body structures and release bound material. Following hydrolysis, equivalent amounts of $CHCl_3$ (50–300 μl) were added and samples were vigorously shaken on a Vortex mixer for 30 sec. Layers were separated by centrifugation at 2000*g* for 3 min. The lower organic phase was filtered, transferred to a vial, and covered with water (10–50 μl). Samples were taken from the lower $CHCl_3$ layer and directly analyzed by GLC-MS.

Isolation of (−)-Palasonin from Seeds of Butea frondosa

Powdered seeds of *Butea frondosa* (140 g) were placed into a Soxhlet apparatus (500 ml) and defatted with low boiling petrol ether (40–60°C, 800 ml) for 24 hr. The seed powder was soaked over night at room temperature in a mixture of *i*-propanol and conc. HCl (500:5 ml). Solids were removed by suction followed by concentration of the solution under reduced pressure and acidification with 2N HCl. Palasonin was thoroughly extracted from the aq. solution with $CHCl_3$ (3×20 ml). After removal of the solvent, the crude brown oil was redissolved in ether (10 ml) and coated onto silica gel (1 g). The solid support was placed into a Soxhlet apparatus (100 ml) and continuously extracted for 16 hr with a mixture of petroleum ether–ether (1:3, v/v). After removal of the solvents, the crude residue (ca. 1.3 g) was recrystallized from low boiling petroleum ether (40–60°C) and yielded 0.47 g of (−)-palasonin. Spectroscopic data and the chiroptical properties (CD and ORD) were in agreement with the literature (Peter et al., 1974).

1-(4-Nitrophenyl)-Ethylimide of (−)-Palasonin

Crystalline (−)-palasonin (0.26 g, 1.42 mmol) isolated from seeds of *Butea frondosa* and (*S*)-(−)-1-(4-nitrophenyl)-ethylamine (0.18 g, 1.08 mmol) were mixed in a small reaction vial (4 ml) and heated to 140°C for 30 min. After cooling, the product was dissolved in CH_2Cl_2 (20 ml) and successively washed with 1N NaOH (15 ml), 1N HCl (15 ml), and water (15 ml). After removal of solvents, the crude product was purified by chromatography on silica gel using a binary

solvent of petroleum ether (40–60°C) and ether (3:5 v/v) for elution. Recrystallization of the condensation product from petroleum ether–ether (3:1 v/v) afforded the imide as colorless needles. Yield: 0.10 g (21%). Mp: 111°C. $[\alpha]_{589}^{26} = 78.8°$ ($c = 0.025$ g/ml, methanol). ^{1}H NMR (acetone-d_6) δ [ppm]: 1.34 (s, 3H); 1.61–1.73 (m, 2H), 1.75–1.83 (m, 1H), 1.79 (d, $J = 7.0$, 3H), 1.95–2.01 (m, 1H), 2.59 (s, 1H), 4.56 (d, $J = 4.9$, 1H), 4.61 (d, $J = 5.5$, 1H), 5.43 (q, $J = 7.1$, 1H), 7.61 (d, $J = 8.85$, 2H), 8.18 (d, $J = 8,85$, 2H). ^{13}C NMR (acetone-d_6) δ [ppm]: 16.1, 17.0, 24.7, 28.9, 49.8, 53.7, 56.7, 81.5, 83.3, 124.2, 128.6, 148.6, 148.5, 177.3, 181.7. IR (KBr) ν [cm^{-1}]: 3050, 1751, 1699, 1516, 1343, 1392, 858. MS (70 eV) *m/z* (%): 330 ($M^{+\bullet}$, 100), 312(20), 300(15), 285(20), 262(60), 244(15), 208(15), 151(60), 113(40), 82(40), 70(60). HR-MS *m/z* [M^+]: calcd. for $C_{17}H_{18}N_2O_5$ 330.121572; found 330.121643.

Crystal Structure Determination

The intensity data for the compound were collected on a Nonius KappaCCD diffractometer, using graphite-monochromated Mo-K$_\alpha$ radiation. Data were corrected for Lorentz and polarization effects, but not for absorption [COLLECT, Data Collection Software; Nonius B.V., Netherlands, 1998 (Otwinowski and Minor, 1997)]. The structure was solved by direct methods (SHELXS; Sheldrick, 1990) and refined by full-matrix least-squares techniques against Fo^2 (SHELXL-97; Sheldrick, 1993). The hydrogen atoms were located by difference Fourier synthesis and refined isotropically. All nonhydrogen atoms were refined anisotropically (Sheldrick, 1993). XP (Siemens Analytical X-ray Instruments, Inc.) was used for structure representations.

Crystal Data for 1-(4-Nitrophenyl)-Ethylimide of (−)-Palasonin

$C_{17}H_{18}N_2O_5$; Mr = 330.33 g/mol, colorless prism, size 0.18 × 0.10 × 0.08 mm^3, monoclinic, space group $P2_1$, $a = 11.3905(8)$, $b = 6.5844(6)$, $c = 12.0322(8)$ Å, $\beta = 117.016(4)°$, $V = 803.9(1)$ Å^3, $T = -90°$C, $Z = 2$, $\rho_{\text{calcd.}} = 1.365$ g/cm^3, μ (Mo-K$_\alpha$) = 1.02 cm^{-1}, $F(000) = 348$, 3134 reflections in $h(-14/14)$, $k(-7/8)$, $l(-15/15)$, measured in the range $3.40° \leq \Theta \leq 27.40°$, completeness $\Theta_{\max} = 99.1\%$, 3134 independent reflections, 2573 reflections with $F_o > 4\sigma(F_o)$, 289 parameters, 1 restraints, $R1_{\text{obs}} = 0.085$, $wR2_{\text{obs}} = 0.112$, $R1_{\text{all}} = 0.115$, $wR2_{\text{all}} = 0.120$, GOOF = 1.149, Flack parameter of 1.7(16), largest difference peak and hole: 0.207/−0.236 e Å^{-3}. Further details of the crystal structure investigation are available on requests from the director of the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK, on quoting the depository CCDC 168993, the names of the authors, and the journal citation.

Detection of Cantharidin and Palasonin and Chiral Analysis

Detection. Five microliters of each beetle extract was injected by using split/splitless injection in a capillary gas chromatograph (GCQ system, Finnigan MAT) equipped with a HT5 fused silica capillary column (bonded phase, SGE; 25 m × 0.32 mm ID, 0.1 μm phase thickness) connected to an ion trap system (GCQ). Electron impact ionization (70 eV) of all samples investigated provided mass spectra with characteristic fragment for palasonin ($M^{+\bullet}$: 182, *m/z* = 114, 82) and cantharidin ($M^{+\bullet}$: 196, *m/z* = 128, 96).

Chiral Analysis. The chiral analysis of palasonin was performed on a TraceMS (Thermoquest, Egelsbach, Germany) equipped with a silica column coated with Lipodex E (Macherey & Nagel, Düren, Germany) (25 m × 0.25 mm, 0.32 μm). The GC injection port was operated at 220°C and the transfer line at 270°C. The oven temperature was programmed from 80°C to 200°C at 5°C/min, with an initial isothermal time of 5 min and a final isothermal time of 10 min. Helium was used as a carrier gas at 3 ml/min and the sample was injected in the splitless mode. Mass spectra were measured in electron impact (EI) mode at 70 eV. Spectra were usually taken in the full scan mode (*m/z* 50–250, one scan per second). In cases of a very low abundance of cantharidin or palasonin, only the two most relevant fragments at *m/z* 82 and *m/z* = 114 were monitored (SIM mode).

RESULTS

Cantharidin and Palasonin in Insects. Blister beetles protect their eggs with cantharidin. The compound is synthesized by the beetles, sometimes only by the males, which transfer it to the females during copulation, for incorporation into the eggs (McCormick and James, 1987). Using both unlabeled cantharidin and stable-isotope-labeled cantharidin combined with mass spectroscopy for analysis, it was demonstrated that such an interindividual cantharidin transfer at copulation and paternal endowment of this toxin into the offspring is a rather common phenomenon among cantharophilous insects (Holz et al., 1994; Eisner et al., 1996). These studies were greatly facilitated by the rather unique mass spectrum of cantharidin, which displays, among others, two highly abundant even numbered fragments at *m/z* = 128 (80%) and *m/z* = 96 (100%), respectively (Figure 1, insert). In continuation of these studies, we recently observed in several specimens of meloid beetles of the genus *Hycleus* minor amounts of another compound that displayed a similar mass spectrum with two major even-numbered fragments that were shifted to lower masses by 14 mass units (Dettner et al., 2001). The compound displayed a quasimolecular ion at $[M+H]^+ = 183$ (cantharidin: $[M+H]^+ = 197$) and two even-numbered fragments at *m/z* = 114 (80%) and *m/z* = 82 (100%). The identity of the fragments, established by high-resolution and linked-scan techniques,

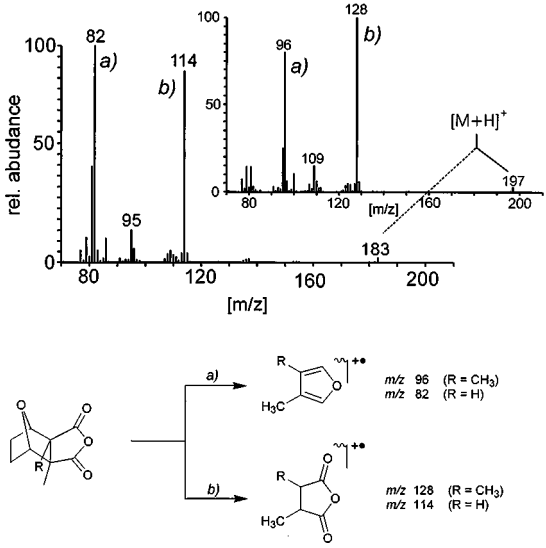


FIG. 1. Mass spectra of cantharidin (insert) and palasonin (main spectrum) from insect extracts. Both compounds are cleaved into the same type of fragments. Methyl-substituted furans (path *a*) and methyl-substituted succinic acid anhydride (path *b*) represent the two most prominent fragments of cantharidin or palasonin.

is outlined in Figure 1 and confirms previous work (McCormick et al., 1986; McCormick and James, 1987).

Due to these characteristic features, the compound was readily identified as palasonin, previously only known to occur in seeds and fruits of the Indian shrub *Butea frondosa*. Within *Hycleus* specimens, there were found 22–321 ng palasonin/mg dry weight (Dettner et al., 2001). Direct comparison of the minor component with authentic palasonin established its identity.

Chiral Analysis of Palasonin. Unlike cantharidin, palasonin is a chiral compound. The lack of the second methyl group introduces dissymmetry and, hence, it is important to know which, and to which extent, individual enantiomers are present in the insects. However, owing to the very low concentration of palasonin in the extracts, only highly sensitive gas chromatographic methods using chiral stationary phases promised to be successful. Optimum results were obtained on an enantioselective capillary column coated with octakis-(3-*O*-butanoyl-2,6-di-*O*-pentyl-γ-cyclodextrin (Lipodex E) that separated racemic palasonin into the two enantiomers with high efficiency ($\alpha = 1.13$).

Figure 2 shows the gas chromatographic separation of palasonin enantiomers from an extract of *Hycleus lunata* Pall a cantharidin-producing meloid. (−)-Palasonin eluted first and the (+) enantiomer second, as established by comparative

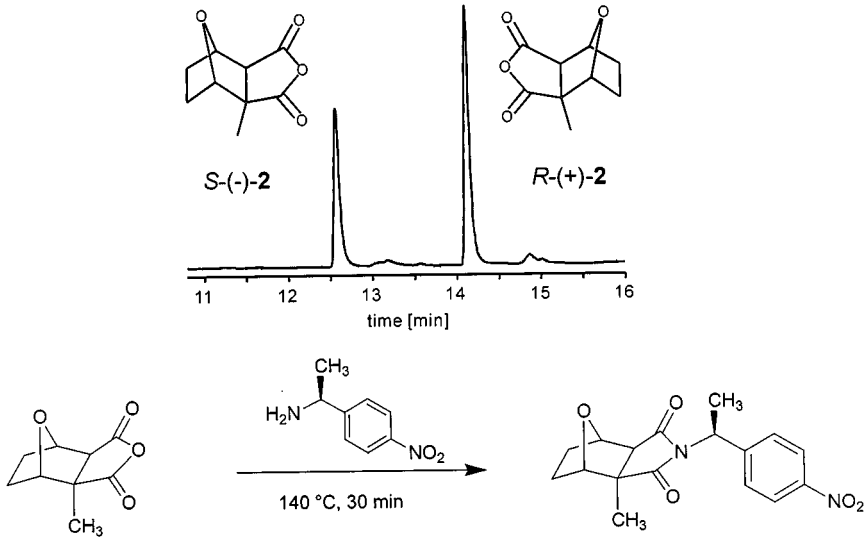


FIG. 2. Gas chromatographic separation of palasonin enantiomers. Palasonin of low ee from an extract of a *Hycleus* sp. is separated on a fused silica column coated with a modified cyclodextrin (Lipodex E). Separation factor: $\alpha = 1.13$.

injection with (−)-palasonin from seeds of *Butea frondosa*. The presence of both enantiomers in the extracts of the meloid beetle *Hycleus lunata* was not unique but proved to be representative for all other hitherto examined palasonin-containing insects. Integration of the peak areas demonstrated that all insect species (Table 1) produced both enantiomers of palasonin with only low to moderate preference for the (+)-enantiomer.

Particularly interesting is the result of the analysis of palasonin from two individuals of the clerid beetle genus *Trichodes apiarius* (L.). One specimen contained almost racemic material, while the other had accumulated (*R*)-(+)-palasonin of 32% ee. Remarkably, this also represents the first record of cantharidin within the beetle family Cleridae. In contrast to palasonin from insects, crude (−)-palasonin obtained from different collections of seeds of *Butea frondosa* proved always to be optically pure (>99% ee).

Absolute Configuration of (+)- and (−)-Palasonin. The absolute configuration of (*S*)-(−)-palasonin from seeds of *Butea frondosa* was originally deduced from its CD spectra and by indirect chemical methods (Peter et al., 1974). The configurational assignment was based on conversion of (−)-palasonin into a secondary alcohol followed by reaction of this intermediate with the anhydride of α-phenylbutyric acid (Horeau, 1961). This empirical method is based on the reaction of secondary alcohols with the above anhydride to give (+)- or

TABLE 1. SPECIMEN ANALYSIS[a]

Toxin origin	Plant or insect species	Cantharidin–palasonin ratio	Palasonin (*S*)-(−) (%)	Palasonin (*R*)-(+) (%)	Palasonin ee (%)
	Leguminaceae				
	Butea frondosa (seeds)	0:100	>99	<1	>98
Cantharidin	Meloidae				
producers	*Hycleus lunata* spec 1	22.4:1	40	60	20
	Hycleus lunata spec 2	205:1	25	75	50
⬇					
Cantharidin	Cleridae				
users	*Trichodes apiarius* spec 1	9.6:1	50	50	0
	Trichodes apiarius spec 1	>99:1	34	66	32
	Staphylinidae				
	Eusphalerum minutum	2.9:1	41	59	18

[a] In *Hycleus lunata* and *Trichodes apiarius* specimens, one individual per sample was analyzed; in *Eusphalerum minutum* four individuals per sample were analyzed.

(−)-α-phenylbutyric acid, depending on the relative size of the substituents flanking the secondary alcohols. Although this indirect method generally proved to be reliable, a direct approach via a crystal structure analysis of a palasonin containing a structural element of known absolute configuration is preferred owing to the unequivocal identification of additional asymmetric centers in the molecule. Palasonin readily reacts with primary amines to give crystalline cyclic imides. A naturally occurring imide of palasonin and (*S*)-(−)-phenylalanin was isolated from seeds of *Butea frondosa* (Barua et al., 1970; Guha et al., 1990). To obtain crystals suitable for structure analysis, we reacted (−)-palasonin (>99% ee; isolated from seeds of *Butea frondosa*) with either (*R*)-(+)- or (*S*)-(−)-1-phenyl-ethylamine. Although the expected imide was formed in good yield, no suitable crystals could be obtained after chromatographic purification. Better results were obtained by reacting (−)-palasonin with 1-(4-nitrophenyl)-ethylamine (Baumgarten and Peterson, 1960) (Figure 3). The additional nitro group in the *p*-position should enhance the

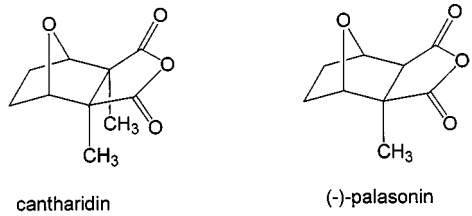


FIG. 3. Reaction of (−)-palasonin from *Butea frondosa* with (*S*)-(−)-1-(4-nitrophenyl)-ethylamine.

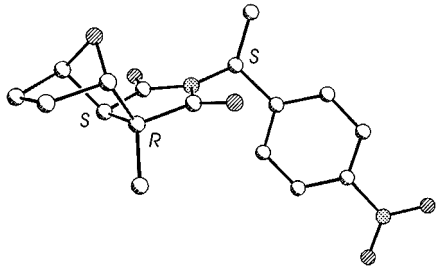


FIG. 4. Molecular structure of the cyclic imide derived from (*S*)-(−)-1-(4-nitrophenyl)-ethylamine and (−)-palasonin as determined by X-ray crystal structure analysis.

tendency of the product to crystallize due to the strongly enhanced intermolecular interactions.

As expected, (−)-palasonin readily reacted with 1-(4-nitrophenyl)-ethylamine to give a cyclic imide that spontaneously crystallized upon removal of the solvent. Recrystallization from a binary mixture of hexane/ether (3:1 v/v) afforded the pure imide. The compound crystallized as monoclinic colorless prisms.

The three-dimensional architecture of the imide is shown in Figure 4. Since the absolute configuration of the asymmetric center next to the aromatic nucleus is known due to the use of (*S*)-(−)-1-(4-nitrophenyl)-ethylamine, the absolute configuration of (−)-palasonin follows as depicted in Figure 4 and unequivocally confirms previous indirect assignments.

DISCUSSION

Cantharidin is an exceedingly powerful toxin that is used by many insects but is only produced by members of two insect families, the meloids and the oedemerids. All canthariphilous and other cantharidin-containing species acquire the toxin from dead or living cantharidin-producing or other cantharidin-containing insects. Specimens of the pollen-feeding staphylinid beetle *Eusphalerum minutum* were regularly found in cantharidin traps, which indicates that they are canthariphilous (Dettner, 1997). Since females of the canthariphilous ceratopogonid *Attrichopogon lucorum* were observed attacking living *E. minutum* by piercing membranes and sclerites of these rove beetles (Klinker, 1983), the presence of palasonin in predatory canthariphilous ceratopogonid flies was expected. Concerning clerid beetles, the search for cantharidin in the genus *Trichodes* hitherto was negative (Juanjie et al., 1995). Therefore, the presence of both anhydrides within certain species of this widespread and abundant beetle family is remarkable. Although it was reported that members of Cleridae are canthariphilous (Bologna and Havelka, 1984; Hemp et al., 1999), specimens of *Trichodes apiarius* were never

observed within cantharidin traps, but occur in various yellow-colored composite blossoms (Dettner, unpublished observations), where they probably feed on cantharidin producing oedemerid and meloid beetles.

Recent analyses of extracts from meloid beetles clearly demonstrated for the first time that palasonin does occur in insects, sometimes even in substantial amounts (Dettner et al., 2001). In general, palasonin was found as a minor or trace compound along with cantharidin as the major toxin (Table 1). As shown by chiral analysis, the insect-derived palasonin is of moderate to very low enantiomeric purity, while the (*S*)-(−)-palasonin from *Butea frondosa* is an optically pure compound. Owing to this major and significant difference between the insect-derived and the plant-produced product, an uptake of palasonin from a hitherto unknown plant source in the environment of meloids and oedemerids appears to be highly unlikely. Hence, it follows that at least the cantharidin-producing meloid beetles must be able to synthesize palasonin *de novo* or by degradation of cantharidin (see below). Moreover, since the various cantharidin-producing specimens of *Hycleus lunata* Pall. generate palasonin with a different ee (Table 1), acquisition of this compound from randomly selected sources by canthariphilous and cantharidin-tolerant predatory insects will inevitably lead to an accumulation of palasonin with varying ee in different individuals of the same species. This phenomenon was observed with two specimens of the canthariphilous genus *Trichodes apiarius* (L.) (Table 1).

Although the mechanistic details of the biosynthesis of cantharidin and/or palasonin are not known, two alternative routes can be envisaged for the origin of palasonin. In the insects, the compound could originate directly from cantharidin (Figure 5, route A) or derive from cyclization of a previously demethylated "12-*nor*-farnesol" (Figure 5, route B) along the pathway analogous to that of cantharidin.

Thus, an oxidative attack onto one of the two angular methyl groups of cantharidin ($-CH_3 \rightarrow -CH_2OH \rightarrow -CHO \rightarrow -COOH$) could finally generate an angular carboxylic acid moiety that, in turn, could readily decarboxylate to palasonin. Owing to the high symmetry of the molecule, a selective functionalization of a distinct methyl group might be difficult and could account for the low ee of palasonin in insects (Figure 5, route A). Alternatively, the oxidative demethylation may occur earlier, on the level of farnesol, the sesquiterpene precursor of cantharidin (Carell et al., 1986) (Figure 5, route B). After oxidative transformation of the two terminal methyl groups into a dicarboxylic acid, one carbonyl group could be lost by decarboxylation. The resulting *nor*- intermediate might be further processed to palasonin, analogous to the pathway to cantharidin. This route might also be operative in seeds or fruits of *Butea frondosa*, since no traces of cantharidin were found along with the main constituent (*S*)-(−)-palasonin. The almost exclusive production of the (*S*)-enantiomer by the plant tissue is also not congruent with the very low ee and the absolute configuration of insect-derived

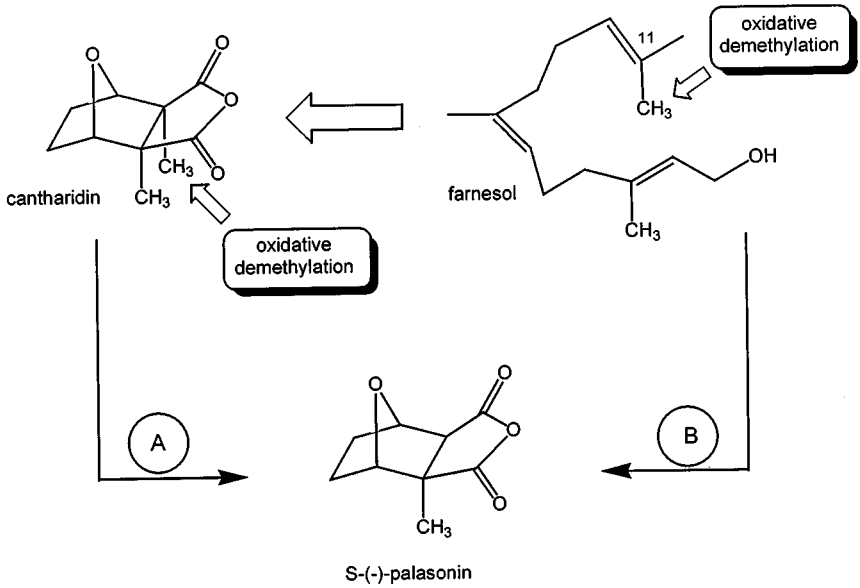


FIG. 5. Alternative biosynthetic routes to palasonin. According to sequence A palasonin is produced by oxidative degradation (demethylation) of cantharidin. Sequence B assumes an early oxidative "demethylation" of farnesol followed by cyclization of the resulting intermediate to palasonin analogous to the biosynthesis of cantharidin (McCormick et al., 1986).

(*R*)-(+)-palasonin and may additionally support an alternative pathway. Although preliminary feeding experiments with larvae of *Oedemera femorata* and synthetic deuterium-labeled 12-*nor*-farnesol precursors demonstrated a successful conversion into labeled palasonin, the amount of the metabolite was too low to assess its configuration and ee (Duetemeyer, 1997; Fietz, 2001). A final decision in favor of a route via late oxidative demethylation (route A) or via demethylation of an early precursor of cantharidin (route B) is, as yet, not possible and awaits further experimental results. Nevertheless, the rather widespread occurrence of palasonin in cantharidin-producing and cantharidin-utilizing insects is unexpected and surprising. Whether or not the compound has a specific ecological function remains to be established and requires further analyses with special emphasis on tissue specific distribution in the insects, its flux between male and female individuals, and, especially, its transfer into eggs for protection of the next generation.

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GUT-BASED ANTIOXIDANT ENZYMES IN A POLYPHAGOUS AND A GRAMINIVOROUS GRASSHOPPER

RAYMOND V. BARBEHENN*

Department of Ecology and Evolutionary Biology
Department of Molecular, Cellular and Developmental Biology
University of Michigan
Ann Arbor, Michigan 48109-1048

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Abstract—Graminivorous species of grasshoppers develop lethal lesions in their midgut epithelia when they ingest tannic acid, whereas polyphagous grasshoppers are unaffected by ingested tannins. This study tests the hypothesis that polyphagous species are defended by higher activities of antioxidant enzymes (constitutive or inducible) in their guts than are graminivorous species. Comparisons were made between four antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APOX), and glutathione transferase peroxidase (GSTPX). Enzyme activities were measured in the gut lumens and midgut tissues of *Melanoplus sanguinipes* (polyphagous) and *Aulocara ellioti* (graminivorous). The results of this study do not support the hypothesis that *M. sanguinipes* is better defended by antioxidant enzymes than is *A. ellioti*, nor are these enzymes more inducible in *M. sanguinipes* than in *A. ellioti* when insects consume food containing 15% dry weight tannic acid. Instead, tannic acid consumption reduced SOD, APOX, and GSTPX activities in both species. This study reports the first evidence that SOD is secreted into the midgut lumen in insects, with activities two- to fourfold higher than those found in midgut tissues. The spatial distribution of GSTPX and APOX activities observed in both species suggests that ingested plant antioxidant enzymes may function as acquired defenses in grasshoppers. In addition, the results of this study permit the first comparison between the antioxidant enzyme defenses of Orthoptera and Lepidoptera. Most notably, grasshoppers have higher SOD activities than caterpillars, but completely lack APOX in their midgut tissues.

Key Words—Grasshopper, Orthoptera, Acrididae, graminivorous, polyphagous, herbivore, *Melanoplus*, *Aulocara*, antioxidant enzyme, superoxide dismutase, catalase, ascorbate peroxidase, glutathione transferase peroxidase.

* E-mail: rvb@umich.edu

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INTRODUCTION

The ingestion of tannic acid by graminivorous grasshoppers (Orthoptera: Acrididae) greatly increases mortality, a result of the formation of ulcerlike lesions through the midgut epithelium (Bernays, 1978; Bernays et al., 1980). By comparison, the consumption of similar amounts of tannic acid by polyphagous grasshoppers produces neither lesions nor increased mortality (Bernays, 1978; Bernays and Chamberlain, 1980; Bernays et al., 1980). The formation of midgut lesions has been attributed to the direct interaction of tannins with the midgut epithelia of grasshoppers (Bernays, 1978) and caterpillars (Steinly and Berenbaum, 1985). Another possible mode of action of ingested tannins is oxidative stress in epithelial tissues. For instance, in some caterpillars the consumption of low-molecular-weight phenols causes elevated levels of oxidative damage to proteins and lipids in midgut tissues (Summers and Felton, 1994; Bi and Felton, 1995), as well as midgut lesions (Lindroth and Peterson, 1988).

Ingested phenolic compounds can act as antioxidants or prooxidants, depending on the physicochemical environment of an insect's gut lumen. If ingested phenols do oxidize, they can produce oxidative stress by two primary mechanisms. The oxidation of phenols produces reactive oxygen species (ROS; Table 1) (Canada et al., 1990), including hydrogen peroxide in the gut lumens of caterpillars (Barbehenn et al., 2001). Hydrogen peroxide is relatively stable and permeates cell membranes (Grisham, 1992). However, its decomposition (via the Fenton reaction) produces highly reactive hydroxyl radicals. Cytotoxicity can result from hydroxyl radical damage to DNA, lipids, and proteins (Hanham et al., 1983; Imlay et al., 1988, Halliwell and Gutteridge, 1999). Secondly, quinones (oxidation products of phenols) may also be cytotoxic and can cause the formation of gut lesions (Gant et al., 1988; Zheng et al., 1997; Thiboldeaux et al., 1998). Ingested tannins appear to oxidize in the gut lumens of grasshoppers (Barbehenn et al., 1996), suggesting that, if antioxidant defenses were inadequate, oxidative stress could produce lesions in the midgut epithelium.

Little is known about the antioxidant enzyme defenses of grasshoppers. Previous work on antioxidant enzymes in insects has been done primarily on caterpillars

TABLE 1. ENZYME-CATALYZED REMOVAL OF REACTIVE OXYGEN SPECIES

Reactive oxygen species[a]	Antioxidant enzyme
$2\,\mathbf{O_2^-} + 2\,H^+ \rightarrow H_2O_2 + O_2$	Superoxide dismutase
$2\,\mathbf{H_2O_2} \rightarrow 2\,H_2O + O_2$	Catalase
$AA + \mathbf{H_2O_2} \rightarrow DHA + 2\,H_2O$	Ascorbate peroxidase
$\mathbf{ROOH} + 2\,GSH \rightarrow ROH + H_2O + GSSG$	Glutathione transferase peroxidase

[a] AA = ascorbic acid, DHA = dehydroascorbic acid, GSH = glutathione, GSSG = glutathione disulfide, ROOH = hydroperoxide.

(e.g., Ahmad et al., 1987; Pritsos et al., 1988; Aucoin et al., 1991; Berenbaum, 1991; Felton and Duffey, 1992; Barbehenn et al., 2001). A variety of antioxidant enzymes protects caterpillar tissues and extracellular fluids from oxidative damage. Among the most widely studied enzymes for detoxifying ROS (including lipid peroxides) are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APOX), and glutathione transferase peroxidase (GSTPX) (Table 1). Two types of evidence have supported the hypothesis that these enzymes serve a defensive role in insect herbivores that feed on plants containing high levels of potential prooxidants. First, antioxidant enzyme activities are high in the tissues of some insect species that feed on such host plants (Pritsos et al., 1988; Lee and Berenbaum, 1990; Aucoin et al., 1991; Felton and Duffey, 1992), and second, in some species, some antioxidant enzymes are induced by ingested prooxidants (Pritsos et al., 1988; Lee and Berenbaum, 1989, 1990; Ahmad and Pardini, 1990; Aucoin et al., 1991; Lee, 1991).

Relatively little attention has been focused on the antioxidant defenses in the gut lumens of insects. Studies on the antioxidant enzymes of caterpillars commonly have examined enzyme activities in whole-body homogenates, providing no information on antioxidant defenses in the gut lumen. However, it is in the gut lumen that ingested phenolic compounds may become extensively oxidized (Barbehenn and Martin, 1994; Barbehenn et al., 1996). Previous work on antioxidant enzymes secreted into the midgut lumens of caterpillars has measured the activities of CAT, APOX, and dehydroascorbate reductase (Felton and Duffey, 1991; Mathews et al., 1997; Barbehenn et al., 2001). This study extends work on antioxidant enzymes to grasshoppers, and examines their activities in both the midgut tissues and gut lumen.

If antioxidant enzyme defenses are strengthened in species of insect herbivores that feed on plants that are rich in potential prooxidants, then polyphagous insects would be expected to have evolved higher antioxidant enzyme activities than have graminivorous insects. Insects that feed on mature grasses ingest far lower allelochemical concentrations, as well as a far lower diversity of these chemicals, compared to insects that feed on dicots (Bernays and Barbehenn, 1987). Secondly, the antioxidant enzymes of polyphagous insects might be more effectively induced by the consumption of potential prooxidants than are those of graminivorous species.

The purpose of this study was twofold: (1) to test the hypothesis that polyphagous grasshoppers are better protected from oxidative stress by antioxidant enzymes, either constitutive or induced, than are graminivorous grasshoppers; and (2) to compare the activities of a suite of antioxidant enzymes in grasshoppers and caterpillars. Two grasshopper species (*Melanoplus sanguinipes* and *Aulocara ellioti*) were compared to test the first hypothesis. *M. sanguinipes* is a polyphagous species in the subfamily Melanoplinae and is tannin tolerant (Bernays et al., 1980). *A. ellioti* is a member of the graminivorous subfamily Gomphocerinae and is

assumed to be tannin sensitive like other graminivorous species examined (Bernays et al., 1980). Four antioxidant enzymes were examined in the gut lumens and tissues of *M. sanguinipes* and *A. ellioti*: SOD, CAT, APOX, and GSTPX. To test the extent of antioxidant enzyme inducibility in the two grasshopper species, enzyme activities were compared after feeding each species control or tannic acid-treated wheat leaves. This study provides the first examination of grasshopper antioxidant enzyme activities, and these values are compared with the enzyme activities from previous studies on larval Lepidoptera.

METHODS AND MATERIALS

Insects

Eggs of *M. sanguinipes* were obtained from a nondiapause colony (USDA, Bozeman, Montana, USA). Insects were reared on romaine lettuce (*Lactuca sativa* var. *longifolia*) and wheat bran as previously described (Barbehenn et al., 1996). Third-instar nymphs of *A. ellioti* were collected in central Wyoming, USA, and reared to adulthood in Ann Arbor, Michigan, USA, in the same conditions as *M. sanguinipes*, with the exception that the host plant was seedling wheat (*Triticum aestivum*). Adult females were used for all experiments, unless otherwise noted. Each experimental insect was placed in a separate ventilated plastic container (470 ml) in an incubator (28°C; 16L:8D) for 2–2.5 days. Insects were assigned at random to feed on control or treated wheat leaves. Seedling wheat leaves were cut and treated with 13 μl of 70% acetone (control) or 13 μl of a 70% acetone solution of tannic acid (Sigma, lot 64F-0049) to give a final concentration of 15% dry weight. Fresh leaves were provided daily, and these were usually completely consumed. In some instances, *A. ellioti* exhibited reduced consumption rates on tannin-treated wheat, and in these cases the level of tannin was reduced to 10% dry wt.

Sample Collection

Each insect was chilled at -20°C for 10 min and dissected. The entire gut was removed from the body, rinsed in distilled water (2–3 sec), blotted dry, and the Malpighian tubules removed. Samples of foregut and midgut contents were weighed in tared microcentrifuge tubes flushed with N_2, and extracted by shaking (10 sec) in N_2-purged buffer containing 50 mg/ml hydrated insoluble polyvinylpolypyrolidone (PVP). The midgut contents included the portion of the food bolus that extended into the anterior hindgut. Extracts of gut contents were incubated for a minimum of 5 min on ice with occasional mixing to remove tannic acid. Midgut tissues, including the caeca, were blotted dry on a paper towel, weighed, and homogenized in N_2-purged buffer (without PVP) in a hand-held

glass tissue homogenizer (20 strokes). Sample extracts were centrifuged (8000*g*, 4° or 22°C, 3–5 min), and kept under a N_2 atmosphere on ice until analyzed. Samples were assayed within 1 hr from the time of collection, unless otherwise noted. Sample sizes are presented in Tables 2–5 below. All buffers were purged of O_2 by bubbling them with N_2 (1 min/ml), unless otherwise noted. Reagents for all assays were purchased from Sigma Chemical Co. or ICN Pharmaceuticals.

Enzyme Assays

Superoxide Dismutase. Dissected gut contents were extracted in 350 μl of sodium phosphate buffer (pH 7.0, 50 mM). Midgut tissues were homogenized in 200 μl of phosphate buffer and centrifuged. Centrifuged extracts were stored at -75°C as they were collected. Thawed supernatant solutions were ultrafiltered to remove low-molecular-weight antioxidants. Sample supernatant solutions (150 μl from gut contents; 100 μl from tissues) were placed in 10,000 molecular-weight cutoff ultrafilters (Millipore Centrifree 0.5). The extracts were reduced to approximately 10 μl by centrifugation of the ultrafilters (8000*g*, 4°C). The retentate (containing SOD) was resuspended with 250 μl of N_2-purged buffer (pH 7.0) and filtered again. The washing process was repeated a second time, after which the retentate was reconstituted to a final volume of 200 μl with phosphate buffer. Sample retentates and SOD standards were stored in microcentrifuge tubes under N_2 at -75°C without loss of activity. Frozen solutions of purified SOD (Sigma) were stable for at least one month. Thawed samples were kept on ice until analyzed. Boiled samples were analyzed to control for nonenzymatic activity. No SOD activity was observed in boiled samples.

The SOD reaction mixture was composed of 145 μl of carbonate buffer (pH 10.2; 62.5 mM with 0.125 mM EDTA), 20 μl of catalase (prepared as 1.3 enzyme units (EU) ml of 50 mM phosphate buffer, pH 7.0), 15 μl of sample extract, and 20 μl of epinephrine (13.6 mM in 100 mM HCl) to start the reaction. The rate of change absorbance at 490 nm was measured with a Biorad Benchmark microplate reader. Kinetic measurements were made using three different dilutions of each sample extract with pH 7.0 phosphate buffer (range = 5–100% of original sample extract concentration). Dilutions were chosen to fall within the linear region in regressions of percent inhibition of the browning reaction versus milligrams of protein in the reaction mixture. One unit of SOD activity was defined as the mass of protein (milligrams) corresponding to a 50% inhibition of the rate of epinephrine oxidation (Misra and Fridovich, 1972; Munkres, 1990). SOD activity was expressed as enzyme units/per milligram of protein. Protein concentrations in the samples were measured with the modified Bradford assay (Stoscheck, 1990), using Bradford reagent and bovine serum albumin as a standard.

Catalase. Samples of foregut and midgut contents from male and female *M. sanguinipes* were extracted in 400 μl of phosphate buffer (pH 7.0, 66 mM).

Tissues were homogenized in 200 μl of buffer. The reaction mixture was composed of 665 μl of potassium phosphate buffer (pH 7.0, 66 mM), 25 μl of sample extract, and 10 μl of 3% hydrogen peroxide. Change in absorbance was measured at 240 nm over a 30-sec period using a Zeiss spectrophotometer. The protein concentration in each extract was measured as described above. CAT activity was expressed as micromoles of hydrogen peroxide reduced per minute per milligram of protein, using an extinction coefficient of 39.4 M^{-1} cm^{-1} (Aebi, 1984). CAT activity in *A. ellioti* was measured as described above, with the exception that females were fed wheat treated with 10% tannic acid (dry wt), 50 mM phosphate buffer was used for extraction and the reaction mix, and 400 μl of buffer was used to homogenize tissues. Rapid bubble formation was observed in cuvettes containing high levels of CAT (e.g., gut tissues), consistent with the formation of O_2 by this enzyme. Negligible CAT activity was observed in boiled samples.

Ascorbate Peroxidase. Samples from the foregut and midgut lumen were extracted in 400 μl of potassium phosphate buffer (pH 7.0, 50 mM) containing 0.5 mM ascorbic acid. Midgut tissues were homogenized in 400 μl of buffer. Samples of seedling wheat (16–21 mg fresh wt) from plants fed to the insects were homogenized in 500 μl of buffer (50 strokes). The reaction mixture was composed of 75 μl of supernatant solution, 646 μl of potassium phosphate buffer (containing 0.5 mM ascorbic acid), and 18 μl of hydrogen peroxide (0.3% in double-distilled water). The rate of change in absorbance at 290 nm was measured with a Zeiss spectrophotometer. Reaction rates were measured in boiled samples (10 min) to correct for nonenzymatic ascorbate oxidation. Protein levels in the samples were measured as described above, and enzyme activity was expressed as micromolar ascorbate oxidized per minute per milligram of protein, using a molar extinction coefficient of 2.8 mM^{-1} cm^{-1} (Asada, 1984). No enzyme activity was measurable using cumene hydroperoxide as the substrate.

Glutathione Transferase Peroxidase. Samples were extracted or homogenized in potassium phosphate buffer (pH 7.0, 50 mM), containing 1 mM EDTA. Foregut and midgut contents were extracted in 150 μl of buffer, and tissues were homogenized in 150 μl of buffer. Wheat leaf samples were collected and homogenized as described above. The reaction mixture consisted of 20 μl of supernatant solution, 20 μl of glutathione reductase (1.67 EU/ml), 100 μl of buffer (pH 7.0, containing 1.0 mM GSH and 0.2 mM NADPH), and 50 μl of cumene hydroperoxide (1.4 mM in buffer) (Ahmad and Pardini, 1990; Weinhold et al., 1990). The rate of decrease in the absorbance at 340 nm was measured for 4 min using a Biorad microplate reader. Boiled samples (10 min) and reaction mixtures lacking certain reagents were assayed as controls. The average reaction rate in boiled samples ($N = 5$–6/type) was subtracted from the rates in active samples to correct for the nonenzymatic oxidation of NADPH. The reaction rate was negligible in the absence of added glutathione or cumene hydroperoxide, and autoxidation of NADPH was negligible in the absence of added samples. Protein levels were

measured as described above, and GSTPX activity was expressed as nmol NADPH oxidized per minute per milligram of protein, using a molar extinction coefficient of 6220 M^{-1} cm^{-1}. Although GSTPX activity was measured promptly in fresh extracts, full activity was recovered after storage at $-75^{\circ}C$ (two weeks).

Statistical Analyses

All analyses were performed using SAS (version 8e; PROC MIXED) (SAS Institute, 2000). Comparisons between means were made using differences of least square means (SAS Institute, 2000).

RESULTS

Antioxidant Enzyme Activities

Superoxide dismutase. Overall, SOD activities were similar in *A. ellioti* and *M. sanguinipes* (Table 2 and Table 6 below). Tannin consumption decreased SOD activity in both species ($P = 0.018$). However, activity levels in the midgut tissues of *M. sanguinipes* were unaffected by tannic acid consumption (94.8% of control levels). By comparison, SOD activities in the midgut tissues of *A. ellioti* were reduced to 73.4% of control levels. SOD activity varied across sites in the gut in a similar fashion within each species: maximal activity was found in the midgut lumen, followed by the foregut lumen, and similar lower levels were found in

TABLE 2. SUPEROXIDE DISMUTASE (SOD) ACTIVITIES IN *Aulocara ellioti* AND *Melanoplus sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID[a]

Species and site	Tannic acid in diet (%)	SOD activity (units/mg protein)	*N*
A. ellioti			
Foregut contents	0	102.7 ± 16.2^{cd}	9
Foregut contents	15	56.6 ± 12.2^{ab}	5
Midgut contents	0	151.2 ± 24.0^{ef}	9
Midgut contents	15	121.0 ± 23.8^{cde}	5
Midgut tissue	0	68.9 ± 4.9^{bc}	9
Midgut tissue	15	50.6 ± 4.7^{a}	12
M. sanguinipes			
Foregut contents	0	88.5 ± 15.1^{bcd}	7
Foregut contents	15	62.8 ± 20.2^{abc}	6
Midgut contents	0	218.2 ± 37.0^{f}	7
Midgut contents	15	137.6 ± 18.4^{def}	8
Midgut tissue	0	57.8 ± 5.2^{ab}	8
Midgut tissue	15	54.8 ± 7.2^{ab}	8

[a] Data are presented as mean $\pm$ SE. Means followed by nonoverlapping letters are statistically different ($P < 0.05$).

TABLE 3. CATALASE (CAT) ACTIVITIES IN *Aulocara ellioti* AND *Melanoplus sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID[a]

Species and site	Tannic acid in diet (%)	CAT activity (μmol/min/mg protein)	*N*
A. ellioti			
Foregut contents	0	26.9 ± 5.5^{a}	9
Foregut contents	15	41.3 ± 7.9^{ab}	10
Midgut contents	0	184.9 ± 32.2^{efg}	9
Midgut contents	15	188.9 ± 15.9^{fg}	9
Midgut tissue	0	182.5 ± 25.3^{defg}	10
Midgut tissue	15	148.0 ± 11.6^{defg}	10
M. sanguinipes			
Foregut contents	0	52.0 ± 9.4^{bc}	7
Foregut contents	15	54.5 ± 7.4^{bc}	8
Midgut contents	0	102.5 ± 28.0^{cd}	9
Midgut contents	15	124.4 ± 27.8^{def}	8
Midgut tissue	0	181.8 ± 42.5^{defg}	10
Midgut tissue	15	235.9 ± 59.7^{g}	9

[a] Data are presented as mean $\pm$ SE. Means followed by nonoverlapping letters are statistically different ($P < 0.05$).

the midgut tissues (Table 2). SOD activities were three- to fourfold higher in the midgut lumens of grasshoppers than in their midgut tissues (Table 2).

Catalase. Overall, CAT activities were similar in *A. ellioti* and *M. sanguinipes* (Tables 3 and 6). CAT activity was unaffected by tannin consumption in both species at each site in the gut (Table 3 and Table 6 below). Within different sites in the gut, however, CAT activity varied between the species: CAT activity in the foregut of *M. sanguinipes* was higher than that in *A. ellioti* ($P = 0.020$), but the reverse was true for CAT activity in the midgut lumen ($P = 0.016$) (Table 3). The greatest CAT activity was present in midgut tissues and midgut lumen contents in *A. ellioti*. The lowest levels of CAT activity were present in the foregut contents, which varied from as little as 14% of midgut lumen levels (*A. ellioti* controls) to 51% of midgut lumen levels (*M. sanguinipes* controls).

Ascorbate Peroxidase. APOX activities were similar in *A. ellioti* and *M. sanguinipes* (Table 4 and Table 6 below). Tannic acid consumption reduced APOX activity ($P = 0.030$) (Tables 4 and 6). This effect was most pronounced in the midgut lumen of *M. sanguinipes*, in which APOX activity in insects fed tannic acid was 21% of control levels (Table 4). A similar treatment effect was observed in *A. ellioti.* APOX activities were substantially higher in the foreguts of both species than in their midgut lumens. No APOX activity was found in the midgut tissues of either species. APOX activity in the seedling wheat leaves consumed by *M. sanguinipes* and *A. ellioti* was 1007.0 ± 128.8 ($N = 3$) and 1017.9 ± 103.3 μM/min/mg ($N = 5$), respectively.

TABLE 4. ASCORBATE PEROXIDASE (APOX) ACTIVITIES IN *Aulocara ellioti* AND *Melanoplus sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID[a]

Species and site	Tannic acid in diet (%)	APOX activity (μM/min/mg protein)	N
A. ellioti			
Foregut contents	0	282.3 ± 49.7^{c}	7
Foregut contents	15	196.7 ± 39.8^{bc}	9
Midgut contents	0	96.0 ± 12.7^{ab}	8
Midgut contents	15	33.6 ± 9.7^{a}	9
Midgut tissue	0	0.0 ± 0.0	8
Midgut tissue	15	0.0 ± 0.0	9
M. sanguinipes			
Foregut contents	0	237.8 ± 31.2^{c}	13
Foregut contents	15	215.8 ± 87.2^{bc}	9
Midgut contents	0	178.4 ± 39.8^{bc}	11
Midgut contents	15	42.3 ± 14.6^{a}	8
Midgut tissue	0	0.0 ± 0.0	8
Midgut tissue	15	0.0 ± 0.0	9

[a] Data are presented as mean $\pm$ SE. APOX activity in wheat leaves consumed was 1007.0 ± 128.8 ($N = 3$) and 1017.9 ± 103.3 ($N = 5$) in experiments on *M. sanguinipes* and *A. ellioti*, respectively. Means followed by nonoverlapping letters are statistically different ($P < 0.05$).

Glutathione Transferase Peroxidase. M. sanguinipes maintained higher GSTPX activities in its foregut and midgut lumens than did *A. ellioti* ($P < 0.001$) (Tables 5 and 6). By comparison, GSTPX activities in the midgut tissues were remarkably similar across species and treatments (Table 5). GSTPX activity in control insects decreased along the length of the gut, dropping over 50% from the foregut to the midgut lumen in both species. Tannin consumption reduced GSTPX activity in both species, most strikingly in the gut lumens. GSTPX activity in the foregut lumen was reduced to 7.2% and 10.4% of control levels in *A. ellioti* and *M. sanguinipes*, respectively. In the midgut lumen, tannic acid consumption reduced GSTPX activities to 39.6 and 78.8% of control levels in *A. ellioti* and *M. sanguinipes*, respectively. GSTPX activity in the seedling wheat leaves consumed by *A. ellioti* was 46.0 ± 11.1 nmol/min/mg ($N = 3$).

Comparison of Orthoptera and Lepidoptera

Table 7 summarizes examples of SOD, CAT, APOX, and GSTPX activities previously measured in caterpillars. Among the quantitative differences between Orthoptera and Lepidoptera, the most striking is the higher activity of SOD in grasshoppers; activities in grasshopper midgut tissues (Table 2) range from 10- to 50-fold greater than in the midgut tissues and whole body homogenates

TABLE 5. GLUTATHIONE TRANSFERASE PEROXIDASE (GSTPX) ACTIVITIES IN *Aulocara ellioti* AND *Melanoplus sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID[a]

Species and site	Tannic acid in diet (%)	GSTPX activity (nmol/min/mg)	*N*
A. ellioti			
Foregut contents	0	11.1 ± 2.8^{cd}	10
Foregut contents	15	0.8 ± 0.5^{a}	9
Midgut contents	0	5.3 ± 1.5^{abc}	10
Midgut contents	15	2.1 ± 1.1^{ab}	10
Midgut tissue	0	15.2 ± 1.0^{d}	10
Midgut tissue	15	10.2 ± 0.8^{c}	10
M. sanguinipes			
Foregut contents	0	28.8 ± 6.9^{e}	9
Foregut contents	15	3.0 ± 1.1^{abc}	10
Midgut contents	0	12.3 ± 3.4^{cd}	9
Midgut contents	15	9.7 ± 2.3^{bc}	9
Midgut tissue	0	15.5 ± 1.6^{d}	10
Midgut tissue	15	10.5 ± 0.6^{c}	10

[a] Data are presented as mean $\pm$ SE. GSTPX activity in wheat leaves consumed by *A. ellioti* was 46.0 ± 11.1 nmol/min/mg ($N = 3$). Means followed by nonoverlapping letters are statistically different ($P < 0.05$).

of caterpillars. Among the qualititative differences that are evident, the most notable is the absence of APOX from the midgut tissues of grasshoppers (Table 4), but its presence in the midgut tissues and whole body homogenates of caterpillars. CAT activities in grasshopper gut tissues and gut fluids (Table 3) are in the range of those found in the midgut tissues and whole-body homogenates of caterpillars (Table 7). APOX activities present in the gut lumens of the grasshoppers in this study also lie within the broad range measured in caterpillars (Tables 4 and 7). GSTPX activities in grasshopper midgut tissues fall at the low end of the range of GSTPX activities found in caterpillar whole-body homogenates (Tables 5 and 7).

The effect of tannic acid consumption on APOX activity in grasshoppers and caterpillars was similar: activity is reduced in the midgut fluids of both Orthoptera and Lepidoptera (*O. leucostigma* and *M. disstria*) (Tables 4 and 7). GSTPX activity is also reduced in grasshoppers and some caterpillars by ingested phenols (tannic acid and quercetin, respectively) (Tables 5 and 7). By comparison, SOD activity is increased in caterpillars by ingested quercetin (Table 7), but is reduced or unaffected by tannin consumption in the examined grasshoppers. CAT activity is unaffected by ingested tannic acid in species of Lepidoptera and Orthoptera (Tables 3 and 7). The specific effects of ingested tannic acid on SOD and GSTPX activities have not been measured in caterpillars.

TABLE 6. PROBABILITY VALUES FROM ANALYSES OF ANTIOXIDANT ENZYME ACTIVITIES IN *Aulocara ellioti* AND *Melanoplus sanguinipes*[a]

Effect	Superoxide dismutase			Catalase			Ascorbate peroxidase			Glutathione transferase peroxidase		
	F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Species	0.26	1, 34	0.614	0.00	1, 35	0.949	0.26	1, 34	0.611	13.37	1, 36	**<0.001**
Site	33.26	2, 34	**<0.001**	38.92	2, 35	**<0.001**	27.76	1, 34	**<0.001**	11.64	2, 36	**0.001**
Treatment	6.18	1, 34	**0.018**	0.55	1, 35	0.464	5.15	1, 34	**0.030**	26.94	1, 36	**<0.001**
Species × site	2.35	2, 34	0.111	8.31	2, 35	**0.001**	1.17	1, 34	0.287	5.82	2, 36	**0.006**
Species × treatment	0.01	1, 34	0.938	0.95	1, 35	0.337	0.00	1, 34	0.960	2.00	1, 36	0.166
Site × treatment	2.28	2, 34	0.117	0.20	2, 35	0.819	0.63	1, 34	0.433	7.72	2, 36	**0.002**
Species × site × treatment	0.90	2, 34	0.417	0.87	2, 35	0.430	1.48	1, 34	0.233	2.21	2, 36	0.124

[a] SAS PROC MIXED analysis. Bold highlights significant cases.

TABLE 7. ANTIOXIDANT ENZYME ACTIVITIES IN LARVAL LEPIDOPTERA[a]

Enzyme and species	Enzyme		Effect of ingested phenol on enzyme activity (phenol)	Reference
	Source	Activity		
Superoxide dismutase				
Trichoplusia ni	whole body	0.9, 2.3 units/mg	Increased (quercetin)	Lee and Berenbaum, 1989; Ahmad and Pardini, 1990
Papilio polyxenes	whole body	2.0, 2.9 units/mg	Increased (quercetin)	Pritsos et al., 1988; Berenbaum, 1991
Papilio glaucus	whole body	1.9 units/mg	ND	Berenbaum, 1991
Depressaria pastinacella	whole body	2.6 units/mg	ND	Lee and Berenbaum, 1990
Anaitis plagiata	whole body	5.5 units/mg	ND	Aucoin et al., 1991
Ostrinia nubilalis	whole body	1.4 units/mg	ND	Aucoin et al., 1991
Manduca sexta	whole body	1.5 units/mg	ND	Aucoin et al., 1991
Lymantria dispar	midgut tissues	5.8 units/mg	Increased (locust compounds)	Peric-Mataruga et al., 1997
Catalase[b]				
Trichoplusia ni	whole body	153, 302 μmol/mg/min	Decreased (quercetin)	Ahmad and Pardini, 1990, Lee and Berenbaum, 1989
Papilio polyxenes	whole body	128, 289 μmol/min/mg[c]	Decreased (quercetin)	Pritsos et al., 1988; Berenbaum, 1991
Papilio glaucus	whole body	73 μmol/min/mg[c]	ND	Berenbaum, 1991
Depressaria pastinacella	whole body	585 μmol/min/mg	ND	Lee and Berenbaum, 1990
Anaitis plagiata	whole body	171 μmol/min/mg	ND	Aucoin et al., 1991
Ostrinia nubilalis	whole body	34 μmol/min/mg	ND	Aucoin et al., 1991
Manduca sexta	whole body	154 μmol/min/mg	ND	Aucoin et al., 1991
Orgyia leucostigma	midgut tissues	611 μmol/min/mg	NS (tannic acid)	Barbehenn, unpublished data
Malacosoma disstria	midgut tissues	802 μmol/min/mg	NS (tannic acid)	Barbehenn, unpublished data
Lymantria dispar	midgut tissues	251 nmol/min/mg	Decreased (locust compounds)	Peric-Mataruga et al., 1997
Orgyia leucostigma	midgut fluid	103 μmol/min/mg	NS (tannic acid)	Barbehenn et al., 2001
Malacosoma disstria	midgut fluid	192 μmol/min/mg	NS (tannic acid)	Barbehenn et al., 2001
Helicoverpa zea	regurgitate	3 μmol/min/mg	ND	Felton and Duffey, 1991

Spodoptera exigua	regurgitate	98 μmol/min/mg	ND	Felton and Duffey, 1991
Heliothis virescens	regurgitate	16 μmol/min/mg	ND	Felton and Duffey, 1991
Ascorbate peroxidase				
Helicoverpa zea	whole body	16, 21 μM/min/mg	ND	Mathews et al., 1997
Orgyia leucostigma	midgut tissue	600 μM/min/mg	Decreased (tannic acid)	Barbehenn unpublished data
Malacosoma disstria	midgut tissue	890 μM/min/mg	NS (tannic acid)	Barbehenn unpublished data
Orgyia leucostigma	midgut fluid	1100 μM/min/mg[d]	Decreased (tannic acid)	Barbehenn et al., 2001
Malacosoma disstria	midgut fluid	1700 μM/min/mg[d]	Decreased (tannic acid)	Barbehenn et al., 2001
Helicoverpa zea	regurgitate	11 μM/min/mg	ND	Mathews et al., 1997
Glutathione transferase peroxidase				
Trichoplusia ni	whole body	50, 200 nmol/min/mg	Decreased (quercetin)	Ahmad and Pardini, 1990; Weinhold et al., 1990
Papilio polyxenes	whole body	253 nmol/min/mg	ND	Weinhold et al., 1990
Spodoptera eridania	whole body	106 nmol/min/mg	ND	Weinhold et al., 1990
Lymantria dispar	midgut tissues	6 nmol/min/mg[e]	NS (locust compounds)	Peric-Mataruga et al., 1997

[a] Data for mid- and final-instar larvae are reported when available. ND = not determined. NS = not statistically significant.
[b] CAT activities are proportional to substrate concentration and may not be completely comparable between studies.
[c] Published in Berenbaum (1991) as mol/mg/min, but presumably is μmol/min/mg.
[d] Note corrected values here.
[e] GSTPX assays used cumene hydroperoxide, with the exception of Peric-Mataruga et al. (1997), which used *t*-butyl hydroperoxide.

DISCUSSION

The results of this study demonstrate that the superior defenses of polyphagous grasshoppers against the effects of ingested tannic acid are not the result of consistently elevated activities of four of the major antioxidant enzymes. Contrary to the main hypothesis, few cases were observed in which *M. sanguinipes* was found to have greater enzyme activities than *A. ellioti*. Among the antioxidant enzymes measured in *A. ellioti* and *M. sanguinipes*, only GSTPX in the foregut and midgut lumen was consistently higher in the tannin-tolerant species. In addition, SOD activities in *M. sanguinipes* gut tissues were unaffected by tannic acid, whereas tannic acid reduced SOD activity in *A. ellioti* tissues. CAT activity was also higher in the foregut lumen of *M. sanguinipes* than in this region of *A. ellioti*. However, the opposite pattern exists for CAT activity in the midgut lumen, where its activity was higher in *A. ellioti* than in *M. sanguinipes*. Enzyme activities in the polyphagous and graminivorous grasshopper species are remarkably similar in many cases, most notably in the tissues of the midgut.

The results of this study also show that antioxidant enzymes are not induced to a greater extent in a polyphagous grasshopper than in a graminivorous species. None of the enzymes examined in either species was significantly induced by tannic acid during a two-day period. On the contrary, lower enzyme activity was associated with tannin consumption, where a significant treatment effect was observed (SOD, APOX, and GSTPX). Ingested tannic acid had a strong inhibitory effect on APOX activity in the midgut fluids of both species. GSTPX activity was also strongly inhibited in the foreguts of both species. Among the enzymes studied, only CAT activity was unaffected by tannic acid in all cases.

In this study it is assumed that the pattern of tannic acid sensitivity observed in examined graminivorous grasshoppers is shared by *A. ellioti* (Bernays et al., 1980). Although this assumption was not tested, an examination of the morphological features found in strictly graminivorous grasshoppers confirmed that *A. ellioti* has both the flattened mandibles and shortened posterior midgut caeca that are typical of graminivorous species (Isely, 1944; Chapman, 1988; Barbehenn, personal observation). Care must also be taken in generalizing from results on the nondiapause strain of *M. sanguinipes*; elevated levels of some detoxification enzymes have been found to arise as artifacts of laboratory rearing in this variety (Isman et al., 1996). The midgut tissues of grasshoppers, particularly those of the midgut caeca, contain the highest detoxification enzyme activities in the body (Feng and Isman, 1994). In this study, it is assumed that these tissues also represent a key site at which the capabilities of antioxidant enzyme defenses can be compared among grasshopper species.

Two distinct sources of antioxidant enzymes in the gut lumens of grasshoppers are apparent: secretion by the midgut and ingestion. The results of this study suggest that luminal SOD and CAT activities are due to enzymes secreted by the

midgut, whereas luminal APOX and GSTPX activities are due to ingested plant enzymes. The spatial pattern of SOD and CAT activity (i.e., higher activity in the midgut lumen than the foregut lumen, and activity in midgut tissues) is consistent with their secretion into the midgut lumen by midgut tissues. The foregut is lined with an impermeable cuticle (Maddrell and Gardiner, 1980), precluding enzyme secretion. However, if antioxidant enzymes were secreted into the midgut lumen, regurgitation into the foregut would provide a source of these enzymes in the foregut. For example, regurgitated gut fluid from the midgut provides digestive enzymes to the foregut (e.g., Evans and Payne, 1964; Ferreira et al., 1990). Secretion of antioxidant enzymes in the saliva cannot be ruled out as an additional enzyme source.

The results of this work also suggest that ingested plant antioxidant enzymes are functional in grasshopper gut fluids. Unlike SOD and CAT, the spatial distribution of GSTPX and APOX activities in the guts of grasshoppers is characterized by maximal activity in the foregut lumen. In the case of APOX, the absence of activity in midgut tissues suggests that it is either inactive at this site or is not synthesized in grasshoppers. On the other hand, the activities of APOX and GSTPX in seedling wheat are sufficiently high to account for their activities in the foreguts of *M. sanguinipes* and *A. ellioti*. APOX activity in wheat is approximately fourfold higher than that found in grasshopper foregut lumens. Similarly, GSTPX activity in wheat leaves ranges from fourfold higher than the maximal (foregut) activity in *A. ellioti* to roughly twofold higher than the maximal (foregut) activity in *M. sanguinipes* (assuming similar enzyme levels across seedling wheat used in separate experiments).

Previous work on plant enzymes ingested by insects has focused on defensive enzymes, such as polyphenol oxidase and guaiacol peroxidase (Duffey and Felton, 1989; Felton et al., 1989; Felton and Duffey, 1991). A considerable body of evidence demonstrates that ingested enzymes also play beneficial roles in the gut lumens of insects. For instance, previous work on ingested fungal enzymes demonstrates that they play an essential role in cellulose digestion in a variety of saprophagous plant-feeding insects (Martin, 1987). The physicochemical conditions in grasshopper gut fluid are conducive to the continued activity of ingested plant enzymes, which function in an acidic pH (ca. 4–6) in the plant (Schultz and Lechowicz, 1986). The pH of grasshopper gut fluid (ca. 6–7) (Barbehenn et al., 1996; Appel and Joern, 1998) is far more favorable for continued plant enzyme activity than the high pH conditions commonly found in caterpillars (ca. 8–12) (Dow, 1984; Appel and Martin, 1990). Do the more extreme conditions in Lepidoptera preclude the acquisition of plant antioxidant enzymes, selecting for the secretion of endogenous APOX in caterpillars? Cofactors necessary for the function of APOX (ascorbate) and GSTPX (glutathione) are present in grasshopper gut fluid in adequate concentrations to permit enzyme activity. Ascorbate is present in the midgut and foregut lumens of both grasshopper species examined at levels

of 300–5000 μM (Barbehenn, in preparation), compared to 500 μM in the APOX assay reaction mixture. Glutathione is present in the midgut and foregut lumens of both species at levels of 200–800 μM (Barbehenn, in preparation), compared to 1000 μM in the GSTPX assay reaction mixture. The potential importance of acquired antioxidant enzymes in insect herbivores needs further work.

If APOX is not present in grasshopper midgut tissues, then what controls hydrogen peroxide levels in these insect tissues? Mathews et al. (1997) propose that CAT is inefficient at reducing hydrogen peroxide to low levels because of its high K_m and that APOX better serves this role. It is possible that CAT could effectively reduce hydrogen peroxide to low levels, despite its high K_m, if high activity levels of this enzyme were produced. However, CAT activities in the grasshoppers examined in this study are not higher than those found in Lepidoptera (Table 7). It is also possible that other, unmeasured enzymes function in grasshoppers and that these are measured in assays intended to measure the degradation of hydrogen peroxide by CAT. This limitation of the assay performed cannot be addressed in this study, although the rapid production of gas bubbles in the "CAT" reaction mixtures is consistent with the activity of CAT.

Neither SOD nor GSTPX has been examined in the gut lumens of Lepidoptera, to my knowledge. SOD would likely be even more useful for catalyzing the dismutation of superoxide in the high pH midguts of larval Lepidoptera; at the high pH of caterpillar gut fluids, the stability of superoxide anion radical is on the order of 100,000-fold greater than in the mildly acidic pH of grasshopper gut fluids (Rosen et al., 1999).

In conclusion, the relative susceptibilities of graminivorous and polyphagous grasshoppers to fatal midgut lesions from ingested tannic acid do not appear to be determined by variation in the activities of the antioxidant enzymes examined in this study. Several alternative hypotheses may be proposed: (1) antioxidant enzymes that were not measured in this study differ between the two species, (2) low-molecular-weight antioxidants are more important than antioxidant enzyme activities, and (3) antioxidant defenses do not explain the relative susceptibility of grasshoppers to develop gut lesions (i.e., a mechanism other than oxidative stress is at work). Additional enzymes that might be examined in grasshopper tissues include ascorbate free radical reductase, quinone reductase, glutathione reductase, and in the lumen and tissues, thioredoxin reductase, dehydroascorbate reductase, and glutathione peroxidase. Low-molecular-weight antioxidants play a critical role in protecting tissues and extracellular fluids from oxidative stress, and compounds such as ascorbate, glutathione, and α-tocopherol could vary among grasshopper species independent of the activities of antioxidant enzymes, such as those measured in this study. In addition, the basis for proposing an antioxidant defense hypothesis to explain the taxonomic variation in grasshopper midgut lesions would be strengthened by an examination of levels of ROS and oxidative stress (biomarkers) in tannin-tolerant and -sensitive grasshoppers.

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DIETARY AND DEVELOPMENTAL INFLUENCES ON INDUCED DETOXIFICATION IN AN OLIGOPHAGE

J. A. CIANFROGNA, A. R. ZANGERL, and M. R. BERENBAUM*

Department of Entomology
University of Illinois at Urbana-Champaign
320 Morrill Hall, 505 S. Goodwin
Urbana, Illinois 61801

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Abstract—Many plant secondary compounds induce detoxification activity in herbivorous insects. Although inducibility may be advantageous as a means of reducing costs associated with maintenance of metabolism, another benefit of inducibility is that it may allow insects to tailor their detoxification profiles to multiple substrate toxins in their diets. The parsnip webworm, *Depressaria pastinacella*, must contend with many types of furanocoumarins, toxins present in abundance in all of its host plants. Previous studies have documented that cytochrome P-450s are responsible for metabolism of furanocoumarins in this species and that this overall activity is inducible. In this study, we examined the effects of ingestion of single furanocoumarins on metabolism of multiple furanocoumarins and the ability of webworms to adjust their metabolism profiles to match artificial diets with furanocoumarin content differing qualitatively and quantitatively from the average content found in their principal host. That detoxification rates of newly molted sixth instars prior to feeding did not differ from those of actively feeding fifth or sixth instars suggests that constitutive activities of furanocoumarin-metabolizing enzymes are maintained in the absence of substrates. All of the induction assays in this study were performed with ultimate instars. Each of the furanocoumarins assayed was found to induce metabolism of five different furanocoumarin substrates; however, the induction profile was independent of the inducing agent. Consistent with this finding, webworms were incapable of matching their detoxification profiles to diets with different furanocoumarin compositions. Thus, the profile of detoxification within individuals of this species appears to be genetically fixed, although there is considerable variation in profiles among individuals.

Key Words—Detoxification, induction, *Depressaria pastinacella*, furanocoumarins, substrate matching, development, cytochrome P-450, oligophage.

* To whom correspondence should be addressed. E-mail: maybe@uiuc.edu

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INTRODUCTION

A wide range of secondary plant compounds induce detoxification activity in herbivorous insects (Brattsten et al., 1977; Cohen, 1989; Nitao, 1989; Snyder and Glendinning, 1996). Insects encounter these secondary compounds, which are produced by plants as a defense mechanism against herbivores (Rosenthal and Berenbaum, 1991), when they ingest plant tissue (Slansky, 1992). Inducibility may enable insects to limit their potential costs of maintaining suites of detoxification enzymes and to tailor their detoxification profile specifically according to their exposure to dietary toxins.

Cytochrome P-450 monooxygenases (P-450s) are heme-based proteins involved in the oxidative metabolism of exogenous and endogenous chemicals. Numerous studies have examined the effects of plant allelochemicals on the induction of P-450 enzymes during larval development of many insects (Ahmad, 1986; Snyder et al., 1995; Stevens et al., 2000). In these studies, P-450 activity was reported to be highest in actively feeding larvae and significantly lower in nonfeeding larvae. Ahmad (1986) and Stevens et al. (2000) noted that P-450 activity was highest when the larvae were mid-stage. Moreover, some allelochemical-metabolizing P-450 genes have in their promoter region sequences showing a high level of identity with ecdysone response elements (Li et al., in preparation), suggesting the possibility of hormonal regulation of allelochemical metabolism. This would be consistent with past observations of adaptive regulation, such as lack of P-450-mediated metabolism activity in nonfeeding stages (egg, pupa, and adult) of *Papilio polyxenes* (Harrison et al., 2001). As well, some insect P-450 genes possess regulatory elements that appear to respond specifically to host-plant allelochemicals (e.g., XRE-xan in *CYP6B1* in *Papilio polyxenes*; Berenbaum, 2002), further suggesting specificity of regulation.

In addition to the potential benefit that inducibility can afford as a cost-saving measure, it may also provide benefits as a means of adjusting detoxification capacity to multiple substrates. Although most herbivorous insects must contend with a suite of secondary compounds that may or may not be biosynthetically related (Berenbaum and Zangerl, 1996), studies of inducibility have typically focused on single secondary compounds or several compounds administered singly (Muehleisen et al., 1989; Snyder et al., 1993; Snyder and Glendinning, 1996). Efficient food utilization by herbivores encountering multiple secondary compounds may necessitate adjustments of multiple detoxification reactions to match intake rates of each secondary compound. Such adjustments have been observed with respect to nutrient digestion. Kamin-Belsky and Wool (1993) tested the effects of different diets on the induction of digestive enzymes in *Ephestia cautella* (Walker) larvae. Ten diets that differed in nutritional composition were fed to larvae, and their ability to induce digestive enzymes was assayed. The pattern of correlations

among the different classes of digestive enzymes was diet-dependent, suggesting that enzyme activities could be modulated independently. In another study, Hinks et al. (1991) demonstrated that adult grasshoppers (*Melanoplus sanguinipes*) that consumed a diet consisting of oats or kochia exhibited a higher level of trypsin and chymotrypsin activity than those fed wheat, which contains more soluble protein than oats or kochia. However, only proteolytic enzymes were assayed, and it is not known whether enzymes for digestion of other substrates (e.g., carbohydrates) were independently modulated.

To date, no studies have been published that document matching of detoxification activities to multiple secondary compounds via the mechanism of independent induction. However, there is suggestive evidence that such a mechanism exists. The oligophagous insect *Depressaria pastinacella* (the parsnip webworm) feeds almost exclusively on wild parsnip (*Pastinaca sativa*), a biennial umbelliferous weed that was introduced from Europe about 300 years ago (Schery, 1972) and is now found throughout eastern North America in a variety of habitats such as roadsides, railroads, open fields, and waste places (Fernald, 1950; Thompson, 1977). The caterpillar, also introduced from Europe, feeds primarily on the fruits and flowers of wild parsnip. In order to utilize wild parsnips, the parsnip webworm must contend with a variety of furanocoumarins, secondary compounds produced mainly by species in the Apiaceae (=Umbelliferae) and Rutaceae that are known for their phototoxic properties (Berenbaum, 1991). Individual wild parsnip plants vary in the relative concentrations of these furanocoumarins, with generally high production of xanthotoxin and bergapten and low production of isopimpinellin and sphondin (Berenbaum and Zangerl, 1998). Webworms rely on cytochrome P-450s to detoxify host plant furanocoumarins (Nitao, 1989). Berenbaum and Zangerl (1998) have shown that webworm populations vary in their ability to metabolize furanocoumarins and that the frequencies of detoxification phenotypes of webworms generally match the frequencies of furanocoumarin chemotypes in the plant population with which they are associated. It has also been shown that furanocoumarin intake has a significant effect on growth rate, nutritional efficiency, and development of *D. pastinacella* (Berenbaum et al., 1989) and that there may be a material cost of detoxification (Berenbaum and Zangerl, 1994).

To determine whether inducibility might serve to reduce detoxification costs in *D. pastinacella*, we tested whether furanocoumarin detoxification rates in *D. pastinacella* larvae decrease following their molt from penultimate to ultimate instar and prior to feeding. To determine whether *D. pastinacella* can adjust their furanocoumarin-metabolizing enzyme levels based on the furanocoumarin content of their diet, we examined furanocoumarin metabolism as a function of larval feeding on diets containing individual furanocoumarins or mixtures of furanocoumarins.

METHODS AND MATERIALS

Postmolt Detoxification Capacity. To determine the effect of a molt on the metabolism of furanocoumarins, fifth- and sixth-instar *D. pastinacella* larvae were collected from a field of wild parsnip plants near a railroad track in west Champaign County, Illinois, USA (5 km NW of the city of Champaign). Fifteen fifth and 15 sixth instars were transferred to plastic cups (29.6 ml capacity; Solo, Urbana, Illinois, USA) with fresh parsnip from the same field. An additional 15 late fifth instars were placed in cups without food to ensure that they would not feed as sixth instars. After 24 hr, the webworms were weighed and dissected in cold sucrose medium (Cohen et al., 1989). The guts were removed, washed of their contents, and homogenized in 0.1 ml of phosphate buffer (pH 7.8, 0.1 M) before being frozen in liquid nitrogen. The phosphate buffer contained PMSF and leupeptin to inhibit protease activity and glycerol to allow the tissue to be frozen without damage. Once all larvae had been dissected, the homogenates were thawed, and 0.7 ml of reaction buffer (phosphate; pH 7.8; 0.1 M), containing a NADPH regenerating system (NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) and (10 μl/0.7 ml) furanocoumarin mixture (with furanocoumarin levels representative of relative proportions normally encountered by the webworms; bergapten = 7.314 μg, isopimpinellin = 6.5 μg, xanthotoxin = 10.36 μg, and sphondin = 3.42 μg), was added to each reaction. Each assay was incubated for 15 min at 30°C, and then 0.2 ml of 2M HCl was added to stop each reaction. To each vial, 0.5 ml of ethyl acetate was added, and each sample was thoroughly vortexed before being centrifuged for 5 min. Approximately 200 μl of the ethyl acetate layer was removed and analyzed by normal-phase high-pressure liquid chromatography (Waters, Milford, Massachusetts, USA, with an Alltech, Deerfield, Illinois, USA, Absorbosphere column, 150 mm long × 4.6 mm ID, 5-μm particle size). The mobile phase consisted of 55% cyclohexane, 42% isopropyl ether, and 3% butanol flowing at 1.5 ml/min and detection was at 254 nm. Samples (20 μl) were automatically injected by a Waters WISP. Metabolism rates were expressed as nanomoles of furanocoumarin metabolized per minute per gram of larva. Statistical analyses were performed using the SPSS statistical package (SPSS, 1999).

Induction by Individual Furanocoumarins. To determine whether individual furanocoumarins differentially induce metabolism of several different furanocoumarin substrates found in wild parsnips, ultimate (sixth) instar webworms from a laboratory colony (constituted from local populations) were fed artificial diets lacking furanocoumarins (Nitao and Berenbaum, 1988) or containing one of the following furanocoumarins at a concentration of 13.9 mM fresh wt: bergapten (0.3% fresh wt), sphondin (0.3% fresh wt) (synthesized by Dr. William Wulff, University of Chicago), psoralen (0.26% fresh wt), and angelicin (0.26% fresh wt) (Indofine, Somerville, New Jersey, USA); all of these naturally occur in its host plants (Murray et al., 1982). The larvae were permitted to feed

for 24 hr, after which they were weighed prior to dissection for metabolism assays.

Webworms were dissected in cold sucrose medium (Cohen et al., 1989), and the guts were removed. After the guts were washed of their contents, they were placed in a 1.5-ml microcentrifuge tube containing 100 μl of phosphate buffer (pH 7.8, 0.1 M). The phosphate buffer contained PMSF and leupeptin to inhibit protease activity. One-fifth of a single gut homogenate, as measured by volume, was used to assay metabolism of each furanocoumarin substrate, thereby allowing the assay of all five furanocoumarin substrates (angelicin, sphondin, bergapten, xanthotoxin, and psoralen) in each larva. The homogenate aliquot was added to 1 ml of reaction buffer (phosphate; pH 7.8; 0.1 M) that contained the NADPH regenerating system (NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) and 10 μg of furanocoumarin. Each assay was incubated for 30 min at 30°C. The remainder of the procedure was the same as described. Metabolism rates were expressed as nanomoles of furanocoumarin metabolized per minute per gram of larva.

Three larvae were assayed per diet. Because rates of metabolism of the five substrates were obtained from each larva, they represent repeated measures and were analyzed by repeated measures analysis of variance with diet as the main between-subjects factor (SPSS, 1999).

Induction by Multiple Furanocoumarins. In order to determine whether webworms can adjust their metabolic profiles to match precisely the phytochemical profile of the food they consume, we first determined the average concentration of furanocoumarins in fruits of *Pastinaca sativa* from four Midwestern populations (using data from Berenbaum and Zangerl, 1998). Averaging across these populations allowed us to estimate an average chemical phenotype, characterized by high levels of xanthotoxin (3 μg/mg), intermediate levels of bergapten (1.5 μg/mg), low levels of isopimpinellin (0.5 μg/mg), and very low levels of sphondin ($<$0.2 μg/mg). We then set out to create furanocoumarin profiles that differed both quantitatively and qualitatively from the average chemical phenotype. One mixture was designed to contain all four furanocoumarins present in the average phenotype but to vary in the proportional representation of each furanocoumarin in the mix. A second mixture was designed to differ qualitatively from the average phenotype in that only half of the compounds in the mixture would be present.

To create these mixtures, furanocoumarins were isolated from seeds of *P. sativa* that were collected in the Phillips Tract, a university-owned natural area 3 km northeast of Urbana, Illinois. The seeds were macerated in a blender with ethyl acetate, and the homogenate was filtered through glass wool and concentrated in a rotary evaporator. For isolation of furanocoumarin fractions, 200 g of thin-layer silica (M. Woelm, Eschwege, Germany) was mixed with hexane to produce a thin slurry. The slurry was then poured into a glass Büchner funnel with a fritted

glass filter. A vacuum was applied to the funnel, and the silica was packed until the hexane level was just at the top surface of the silica. A filter paper disk was placed on top of the silica to ensure that the surface would not be disturbed by addition of the sample or elution solvents. Hexane was added to the concentrated extract until the solution became flocculent (furanocoumarins are not readily soluble in hexane). The extract was then poured onto the filter paper and loaded into the top layer of silica with additional hexane. A mixture of cyclohexane (55%), isopropyl ether (42%), and *n*-butanol (3%) was used to separate the furanocoumarins from other constituents, and the resolving bands were visualized with the aid of a short-wave ultraviolet lamp, which causes the furanocoumarins to fluoresce. By collecting fractions at different stages of the elution, extracts were produced with different furanocoumarin profiles. These fractions were analyzed by normal-phase HPLC as described above. Fractions were consolidated in different amounts to produce two distinct furanocoumarin mixes. One mix, the qualitatively different mix, contained large amounts of bergapten and xanthotoxin with effectively no isopimpinellin or sphondin. The other mix, the quantitatively different mix, contained all four furanocoumarins, but in proportions different from the average phenotype: approximately equivalent concentrations of all four furanocoumarins ($1\ \mu$g/mg $\pm$ $0.5\ \mu$g/mg) (Figure 1).

To ensure a wide range of genetic variation, webworms were collected as pupae from four populations in central Illinois (two in Kankakee County, one in Champaign County, and one in Douglas County) and maintained as separate populations according to the methods of Berenbaum and Zangerl (1998) to obtain fifth instars to be reared on a semidefined artificial diet lacking furanocoumarins. Two artificial diets were prepared. The first contained 0.1% (fresh wt) of the qualitatively different furanocoumarin mixture and the second contained 0.1% (fresh wt) of the quantitatively different furanocoumarin mixture. Each sixth instar was weighed and placed into a plastic cup (29.6 ml capacity; Solo). A preweighed amount of solidified diet (mean weight = 0.731 g) was added to the cup. A total of 50 webworms were placed on each diet type and allowed to feed for two days. Care was taken to ensure that equal numbers of larvae from each population were represented in each treatment. After feeding, the larvae were weighed, and the remaining diet and frass were dried completely in a desiccator before their mass was recorded. As a standard, an uneaten diet sample was dried down in a desiccator to determine the wet to dry mass conversion. These values were used to obtain information on performance of the larvae on the two different types of diets with respect to growth rates and efficiency of utilization.

After feeding on the diets for two days, the webworms were dissected as described above, except that the guts were placed in phosphate buffer containing glycerol (20%) to allow the tissue to be frozen in liquid nitrogen and stored at -80°C. The guts were later thawed 10 at a time on ice and homogenized in the microcentrifuge tube with a glass pestle, refrozen in liquid nitrogen, and stored

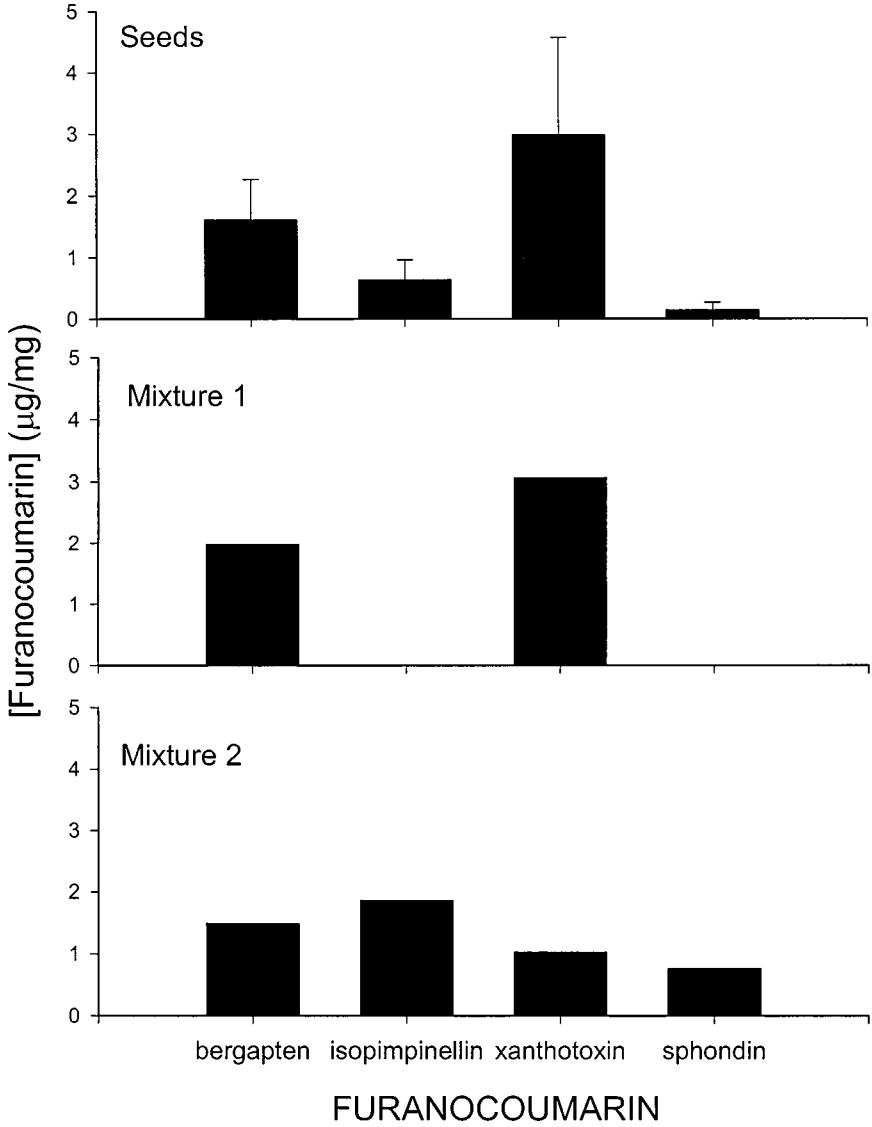


FIG. 1. Furanocoumarin concentrations of artificial diet treatment diets and seeds of wild parsnip.

at −80°C until the metabolism assays were performed. A preliminary experiment confirmed that the freezing, thawing, homogenization, and refreezing of the sample do not affect the rates of furanocoumarin metabolism measured.

The homogenates were allowed to thaw at room temperature. Once thawed, 1 ml of reaction buffer containing a mixture of furanocoumarins (10 μg/ml), representative of relative proportions normally encountered by the webworms, was added (bergapten = 7.314 μg, isopimpinellin = 6.5 μg, xanthotoxin = 10.36 μg, and sphondin = 3.42 μg). The assay was otherwise performed as described above, except that the entire gut homogenate was used in a single assay and the reaction was run for 25 min. Metabolism rates were expressed as nanomoles of furanocoumarin metabolized per caterpillar.

Dry diet samples and dry frass samples (three from each treatment) were randomly chosen for analysis of unmetabolized furanocoumarins. The diet samples were ground to a powder using a modified Wig-L-Bug (Dental Manufacturing Co., Chicago, Illinois, USA). To each diet sample 1 ml of ethyl acetate was added, and to each frass sample 0.5 ml of ethyl acetate was added. These samples were allowed to extract in solvent 24 hr before being analyzed by HPLC as described above.

The effects of diet on growth, consumption, and food utilization efficiency were examined by performing analysis of covariance according to Raubenheimer and Simpson (1992). To determine whether diet type altered furanocoumarin metabolism, we performed a repeated measures analysis of variance with diet and webworm population origin as main effects and metabolism rates of each of the four furanocoumarins as the within-subjects repeated measures effect. Population origin was not significant in this model and so a reduced model combining populations was also evaluated. Unmetabolized furanocoumarins passing into the frass were compared to rates of metabolism to determine whether there was a correlation between the two variables. To determine which factors among initial larval mass, consumption, and metabolism influence final larval mass on each diet, we performed multiple regressions with furanocoumarin metabolism expressed in nanomoles per minute. All statistical analyses were performed using the SPSS statistical package (SPSS, 1999).

RESULTS

Postmolt Detoxification Capacity. Metabolism of xanthotoxin and sphondin did not differ among feeding fifth instars, feeding sixth instars, and freshly molted sixth instars (Figure 2; $P = 0.581$ for xanthotoxin and $P = 0.933$ for sphondin). Thus, overall detoxification activity is not reduced in *D. pastinacella* during the intermolt period.

Induction by Individual Furanocoumarins. All four of the furanocoumarins administered individually in the diet increased rates of furanocoumarin metabolism (Figure 3). Angelicin metabolism was negligible in larvae fed control diet, making

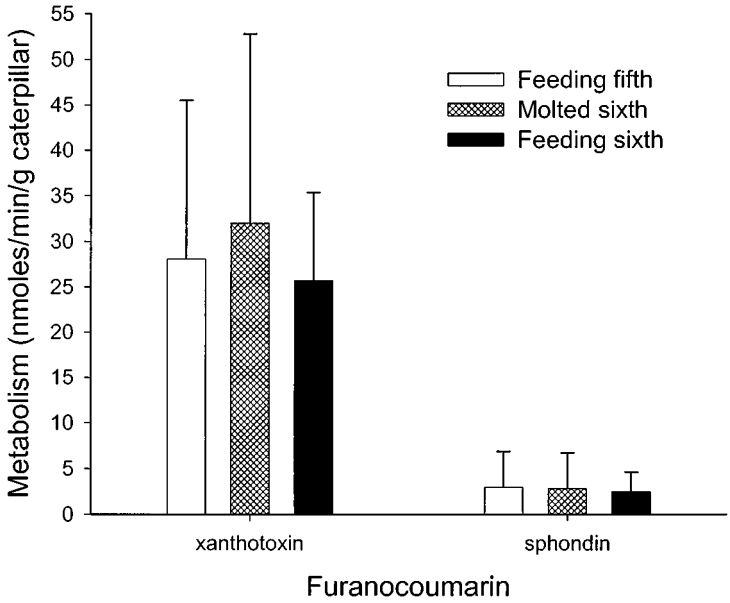


FIG. 2. Mean metabolism rates (+SD) of xanthotoxin and sphondin in feeding fifth-instar larvae, freshly molted sixth-instar larvae, and feeding sixth-instar larvae of parsnip webworms ($N = 15$ for each stage). There were no significant treatment differences for metabolism of xanthotoxin (ANOVA, $df = 2,42$; $F = 0.551$; $P < 0.581$) or sphondin (ANOVA, $df = 2,42$; $F = 0.069$; $P = 0.933$).

calculations of proportional increase impossible. For the other furanocoumarins, induction was as much as 27-fold (for angelicin induction of sphondin metabolism) or as little as 0.6-fold (for sphondin induction of psoralen metabolism). Repeated-measures analysis of variance (Table 1) confirmed that diet type significantly affects furanocoumarin metabolism and that webworms metabolize the furanocoumarins at different rates (significant within-subjects effect, $P < 0.001$). The interaction between diet type and metabolism ($P = 0.005$) suggests that not only is furanocoumarin metabolism affected by diet, but the rates of metabolism of the different furanocoumarins relative to one another are also altered by diet. This effect, however, appears to result solely from the difference between control and furanocoumarin diets, as the interaction disappears when the data from the control diet are omitted from the analysis (interaction P in that model is 0.114). Overall, rates of metabolism were highest for psoralen, intermediate for xanthotoxin and bergapten, and lowest for angelicin and sphondin.

Induction by Multiple Furanocoumarins. The effects of the two different diet types with a mixture of furanocoumarins were indistinguishable with respect to

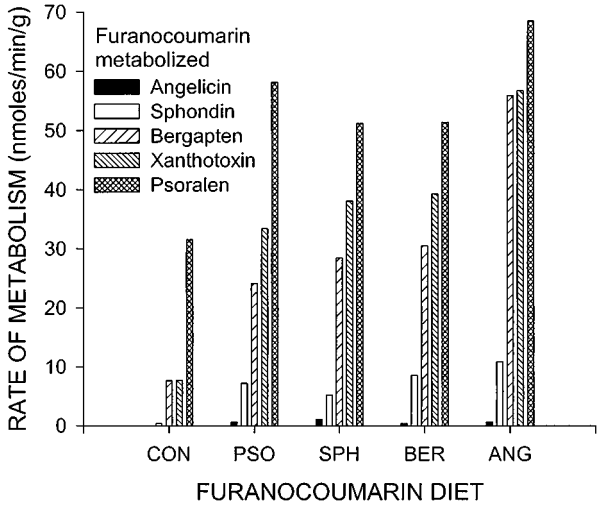


FIG. 3. Mean rates of metabolism of angelicin, sphondin, bergapten, xanthotoxin, and psoralen by parsnip webworms fed diets containing a single furanocoumarin or a diet containing no furanocoumarins ($N = 3$ for each diet).

growth and feeding (Table 2). Analyses of covariance (Table 3) suggest that final larval mass was not affected by diet type with initial larval mass as a covariate and that larval mass gain was not affected by diet with consumption or net consumption (consumption minus frass) as a covariate. An effect of diet type on consumption

TABLE 1. REPEATED-MEASURES ANALYSIS OF VARIANCE OF DIET-INDUCED FURANOCOUMARIN METABOLISM IN *Depressaria pastinacella*[a]

Factors	Sums of squares	*df*[b]	*F*	*P*
Between-subject effects				
Diet type	6,351	4	12.56	0.001
Error	1,265	10		
Within-subject effects				
Metabolism	26,923	2.002	133.5	<0.001
Metabolism × Diet type	3,340	8.006	4.14	0.005[c]
Error	2,017	20.016		

[a] Diet type was a main between-subjects effect and metabolism of each of the five furanocoumarin substrates was a repeated (within-subjects) effect.

[b] The assumption of sphericity was not met in these data; therefore, adjustments to the degrees of freedom were made, and the Greenhouse-Geisser tests are reported (SPSS, 1999).

[c] This interaction becomes nonsignificant ($P = 0.114$) when the control is dropped from the analysis.

TABLE 2. MASS OF *Depressaria pastinacella* LARVAE BEFORE AND AFTER FEEDING AND ABSOLUTE AND NET AMOUNTS (MINUS FRASS) OF DIET CONSUMED

Variable	Diet mixture 1	Diet mixture 2
Larval mass (fresh wt, mg)		
Initial	30.4 ± 6.0	32.6 ± 6.9
Final	75.0 ± 9.4	74.9 ± 9.9
Diet consumed (dry wt, mg)	36.6 ± 5.7	36.0 ± 6.7
Net diet consumed (dry wt, mg)	17.7 ± 3.2	17.2 ± 3.9

[a] Larvae fed for two days on diet mixtures containing furanocoumarins. Values are mean ± SD. Analyses of covariance involving these measures are presented in Table 3.

was observed, however, with larvae eating less of the diet containing all four furanocoumarins (adjusted means were 35.3 mg vs. 37.4 mg). In the absence of dietary sphondin and the near-absence of isopimpinellin, regressions of larval final mass versus initial mass, consumption, and metabolism (Table 4) yielded significant relationships for all three independent variables. For larvae fed the diet containing all four furanocoumarins, however, only consumption and metabolism were predictive of final larval mass.

TABLE 3. ANALYSIS OF COVARIANCE OF DIET TYPE EFFECTS ON GROWTH AND FEEDING EFFICIENCY OF *Depressaria pastinacella*[a]

Source	Sums of squares	*DF*	*F*	*P*
Dependent variable: Mass gain				
Diet type	23.0	1	1.86	0.176
Food consumption	175	1	14.2	<0.001
Error	1098	89		
Dependent variable: Mass gain				
Diet type	21.7	1	1.71	0.194
Net food consumption	145	1	11.5	0.001
Error	1128	89		
Dependent variable: Food consumption				
Diet type	0.0001	1	4.71	0.033
Initial larval mass	0.00162	1	76.2	<0.001
Error	0.00189	89		
Dependent variable: Final larval mass				
Diet type	0.000103	1	1.81	0.182
Initial larval mass	0.00336	1	58.90	<0.001
Error	0.00508	89		

[a] The hypothesis that slopes were homogeneous in each of the four analyses was not rejected (all $P > 0.402$).

TABLE 4. MULTIPLE REGRESSION OF RELATIVE CONTRIBUTIONS OF INITIAL MASS, CONSUMPTION RATES, AND COMBINED FURANOCOUMARIN METABOLISM TO FINAL LARVAL MASS (DEPENDENT VARIABLE) OF *Depressaria pastinacella* ON TWO DIET TYPES

	Regression Coefficients b	SE	Standardized regression coefficient (β)	t	P
Qualitatively different diet					
Initial larval mass (g)	0.497	0.214	0.316	2.32	0.026
Food consumption (g)	0.767	0.228	0.426	3.36	0.002
Metabolism per larva (nmol/min)	0.00387	0.001	0.277	2.96	0.005
Quantitatively different diet					
Initial larval mass (g)	0.162	0.173	0.113	0.936	0.354
Food consumption (g)	0.878	0.174	0.595	5.06	<0.001
Metabolism per larva (nmol/min)	0.00422	0.002	0.264	2.71	0.009

Repeated measures analysis (Table 5) revealed a significant effect of diet type, with diet containing only two of the furanocoumarins eliciting higher overall rates of metabolism. The analysis also yielded a significant repeated measures effect (i.e., the metabolism rates were different among the four furanocoumarins). The lack of a significant interaction, albeit only marginally nonsignificant, between diet and the repeated measure ($P = 0.06$), however, indicates that the webworm larvae were unable to adjust their metabolism profiles to match what they consumed. That this interaction was close to significant suggests that there may have been

TABLE 5. REPEATED-MEASURES ANALYSIS OF VARIANCE OF DIET-INDUCED FURANOCOUMARIN METABOLISM IN *Depressaria pastinacella* WITH DIET TYPE AS MAIN BETWEEN-SUBJECTS EFFECT AND METABOLISM OF EACH OF FOUR FURANOCOUMARIN SUBSTRATES AS REPEATED (WITHIN-SUBJECTS) EFFECT

Factors	Sums of squares	df[a]	F	P
Between-subject effects				
Diet type	88.4	1	5.55	0.021
Error	1434	90		
Within-subject effects				
Metabolism	11942	1.33	1562	<0.001
Metabolism × diet type	25.2	1.33	3.29	0.060
Error	688	120		

[a] The assumption of sphericity was not met in these data; therefore, adjustments to the degrees of freedom were made appropriate to the Greenhouse-Geisser (SPSS, 1999).

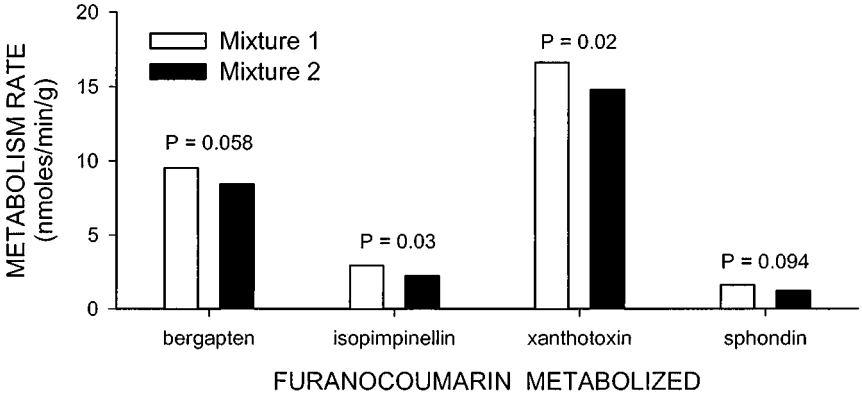


FIG. 4. Mean metabolism rates of bergapten, isopimpinellin, xanthotoxin, and sphondin in parsnip webworms fed a diet containing a mixture of furanocoumarins qualitatively different from that encountered in nature (essentially lacking isopimpinellin and sphondin, $N = 44$) and a diet containing a mixture of furanocoumarins similar to that encountered in the host plant but lower in overall quantity (wild parsnip) ($N = 48$).

a slight degree of adjustment, but it is clear from the comparison between what was eaten (Figure 1) and the differences in metabolism profiles (Figure 4) that any such adjustment was small. Moreover, this inability to adjust metabolism seems to be geographically widespread; a similar repeated-measures model including also webworm population origin (as a fixed main effect) yielded no effect of population nor any interaction of population with the repeated measure or diet (all $P > 0.5$).

The difference in furanocoumarin content that was ingested as diet and eliminated in the frass was correlated with the metabolism rate of individual furanocoumarins in only two instances. As might be expected, the decline in bergapten between its ingestion and elimination in frass was positively correlated with bergapten metabolism ($r = 0.339$, $P = 0.018$, $N = 48$), but it was also correlated with xanthotoxin metabolism ($r = 0.404$, $P = 0.004$, $N = 48$). These relationships held only for larvae feeding on the quantitative diet, which contained all four furanocoumarins. The lack of correlation between metabolism rates and disappearance rates for other furanocoumarins is likely due to imprecision in quantifying furanocoumarins in diet and frass samples, which are not entirely homogeneous.

DISCUSSION

Previous studies have shown that enzymatic activity increases from one larval stage to the next, with a significant decline right before molting. The results from this study indicate that metabolism rates in actively feeding *D. pastinacella*

remain constant from fifth to sixth instar and do not decline between stages. This maintenance of rates prior to feeding that are indistinguishable from those of actively feeding larvae suggests that, if there are costs of detoxification, they are not sufficient to warrant down-regulation. A possible explanation as to why metabolic activity does not change during the intermolt period is that webworms are never in a situation where they are in an environment free of furanocoumarins. Because of the specialized feeding preferences and sedentary habits of the parsnip webworm, which spins a silken web within the plant reproductive structures on which it feeds, the caterpillar is in constant contact with high levels of furanocoumarins not only through ingestion but also possibly through cuticular absorption (e.g., for black swallowtails, see Petersen et al., 2001). In fact, larvae fed diets lacking furanocoumarins nevertheless maintain rates of furanocoumarin metabolism that are high relative to other species not specialized on furanocoumarin-containing plants (Berenbaum and Zangerl, 1994). Reducing rates of furanocoumarin metabolism may thus expose webworms to the toxic effects of their hostplants.

Inducibility is unlikely to function as a means to tailor detoxification rates to dietary intake. Sixth-instar parsnip webworms did not adjust their pattern of metabolism to one particular furanocoumarin or to a mixture of furanocoumarins in their diet. Diet type had no significant effect on the rates of metabolism of furanocoumarins relative to each other but did affect overall rates of metabolism. This lack of a significant interaction suggests that the dietary furanocoumarins tested do not differentially induce furanocoumarin metabolism, a result that is consistent with the finding that webworms cannot adjust their metabolism profiles to match precisely what they are eating, irrespective of whether the furanocoumarin levels differ quantitatively or qualitatively from average host-plant levels.

That the addition of furanocoumarins to artificial diet does not alter the webworm's pattern of metabolism suggests that this trait may be largely genetically fixed in an individual. That is not to say that the trait is uniform among individuals, although correlations between metabolism rates of the substrates were all significant ($P \ll 0.05$) and positive. Xanthotoxin and bergapten metabolism rates were highly correlated ($r = 0.961$), but correlations between rates of metabolism of these two substrates and isopimpinellin ($r = 0.79$ and 0.857, respectively) were lower and were lower yet with sphondin ($r = 0.568$ and 0.577, respectively), suggesting substantial variation in metabolism profiles among individuals. The variation among individuals may be due to the presence of alternative alleles in populations that code for enzymes with differing metabolism rates and substrate specificities. That distinct P-450 enzymes occur in webworm populations is indicated by the less than perfect correlations between metabolism rates of some of the furanocoumarins, the exception being the correlation between xanthotoxin and bergapten metabolism. The lack of flexibility within individuals might occur if the genes coding for the furanocoumarin-metabolizing P-450s share the

same regulatory apparatus. The fact that any one of the furanocoumarins found in webworm hosts can induce metabolism of all of the furanocoumarins lends credence to the possibility at least of a common regulatory response element. Alternatively, the level of dietary furanocoumarins administered, albeit within the range of what the insect encounters in its hosts, may have been sufficiently high to induce maximum capacity for all of the substrates; if that were the case, exposure to lower levels of furanocoumarins might have produced differences in metabolism profiles.

The results from these experiments suggest that individual webworms can adjust their furanocoumarin metabolism quantitatively, but not qualitatively. There may be limited genetic variation for induction in extant webworm populations that restricts individuals to specific patterns of metabolism. The initial population of webworms that was introduced from Europe may have represented only a small proportion of the entire range of ancestral phenotypes; whether independent induction occurs in ancestral European populations remains an open question.

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EFFECTS OF FURANOCOUMARINS ON FEEDING BEHAVIOR OF PARSNIP WEBWORMS *Depressaria pastinacella*

J. A. CIANFROGNA, A. R. ZANGERL, and M. R. BERENBAUM*

Department of Entomology
University of Illinois at Urbana-Champaign
320 Morrill Hall, 505 S. Goodwin
Urbana, Illinois 61801

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Abstract—The parsnip webworm, *Depressaria pastinacella*, exhibits limited physiological resistance to furanocoumarin toxins in its principal host, the wild parsnip, *Pastinaca sativa*. These insects are typically found attacking individual plants low in furanocoumarins, relative to others within populations. They also feed preferentially on parthenocarpic fruits, which are lower in furanocoumarin content than are normal fruits. However, in a previous study with artificial diets, they did not appear to discriminate between high and low concentrations of furanocoumarins. In this study, the ability of webworms to distinguish between diets differing in furanocoumarin content was examined with an artificial diet containing wild parsnip and in green parsnip fruits with and without supplemental furanocoumarins. Larvae showed no preference for high or low furanocoumarin diets containing equal amounts of freeze-dried parsnip fruit powder. When given a choice between otherwise similar wild parsnip fruits, webworms strongly preferred fruits that were not augmented with furanocoumarins in one plant but showed no preference or only a weak preference for nonaugmented fruits in four other plants. In order to identify chemical constituents other than furanocoumarins that might determine feeding preferences, we compared the chemical profile of parthenocarpic fruits (which are preferred) to that of normal fruit. Octyl butyrate, a known deterrent to webworms, is highly correlated with furanocoumarin content, occurs in all plants, and differs significantly among normal and parthenocarpic fruit, suggesting that webworms may be able to avoid furanocoumarins by virtue of their behavioral response to octyl butyrate.

Key Words—Behavior, furanocoumarins, insect, parsnip webworm, *Depressaria pastinacella*, wild parsnip, *Pastinaca sativa*.

* To whom correspondence should be addressed. E-mail: maybe@uiuc.edu

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INTRODUCTION

Specialist herbivores generally feed on a limited number of host plants, often within a single family (Bernays and Graham, 1988) and, therefore, tend to encounter a narrow range of plant secondary compounds. Because specialist herbivores typically do not encounter the variety of secondary plant compounds that generalist herbivores do, they may lack the discriminative feeding behaviors that allow insects with a broader diet range to choose among plants (Bernays and Chapman, 1994).

Depressaria pastinacella, the parsnip webworm, is a specialist herbivore that feeds preferentially on the fruits and flowers of *Pastinaca sativa*, the wild parsnip (Zangerl and Berenbaum, 1993). *D. pastinacella* encounters a suite of furanocoumarins while feeding on the fruits and flowers of *P. sativa*, and the concentration of the furanocoumarins varies among plants as well as within individual umbels (Berenbaum and Zangerl, 1986, 1998). Within wild populations of *P. sativa*, *D. pastinacella* successfully attacks individual plants with lower amounts of furanocoumarins, suggesting either an ability to detect and avoid high concentrations of these chemicals or early differential mortality (Zangerl and Berenbaum, 1993). Ovipositing moths appear to be incapable of distinguishing among hosts based on suitability for their offspring, instead preferentially laying their eggs on larger plants (Zangerl and Berenbaum, 1992). The most conspicuous selective feeding behavior in parsnip webworms involves larval preference for parthenocarpic fruits (fruits without viable seeds). These fruits are preferred despite their low nutritional value compared to normal fruits (Zangerl et al., 1991). Zangerl et al. (1991) reported that between 2.5 and 13 empty fruits were consumed for every filled fruit consumed, depending on the plant from which fruits were taken. They also found that the number of empty fruits that the webworms consumed is negatively correlated with feeding efficiency and growth rate. This decrease was attributed to the low nutritional value of parthenocarpic fruits; filled fruits are 1.6 times higher than parthenocarpic fruit in nitrogen and caloric content, but they are also higher in their furanocoumarin content. These results suggest that webworms prefer parthenocarpic fruits because of their reduced furanocoumarin content, but it is still unclear whether furanocoumarins or other chemical constituents directly mediate this feeding choice.

If preference by *D. pastinacella* larvae is governed directly by furanocoumarin content, it was not apparent in an earlier artificial diet study (Berenbaum and Zangerl, 1992). Sixth-instar *D. pastinacella* larvae that were given the choice between artificial diet supplemented with high and low levels of xanthotoxin, bergapten, sphondin, or a mixture of all three furanocoumarins showed no preference. Moreover, they did not show an ability to discriminate between these diets even when there was a fourfold difference in furanocoumarin level (Berenbaum and Zangerl, 1992). One possible explanation for this lack of discriminatory behavior is that, due to the variation in furanocoumarin concentration that *D. pastinacella* encounters, caterpillars may not invest as much energy in discriminative behavior

because their metabolic capabilities may be more important in determining their interaction with their host plant. Alternatively, the bioassay results may be misleading.

A problem with the study conducted by Berenbaum and Zangerl (1992) is that they tested the effects of only three furanocoumarins and only in artificial diet. There are at least seven furanocoumarins present in green tissues of wild parsnip (Berenbaum and Zangerl, 1996), and there may be interactions among furanocoumarins that provide cues for webworms to consume a particular diet. Alternatively, the inability of webworms to discriminate between high and low furanocoumarin levels reported by Berenbaum and Zangerl (1992) may be due to a failure to consider responses to other chemicals, correlated with furanocoumarins, acting as kairomones. Indeed, Carroll and Berenbaum (in press) demonstrated that octyl acetate, correlated with furanocoumarin content in fruits (Carroll et al., 2000), is an attractant for parsnip webworms. Moreover, components of artificial diets can themselves influence the feeding behavior of caterpillars toward allelochemicals (Shields and Mitchell, 1995; Huang and Renwick, 1997).

In this study, we determined whether webworms can differentiate between artificial diets with high and low furanocoumarin content in a matrix of freeze-dried plant material and between parsnip fruits with and without supplemental furanocoumarins. We also compared chemical components other than furanocoumarins in preferred and nonpreferred plant tissues to determine if other chemicals may act as kairomones.

METHODS AND MATERIALS

Choice between Plant-Containing Artificial Diet with High or Low Furanocoumarin Content. Furanocoumarin assessment by webworms may require the presence of other plant constituents. To test this possibility, we presented webworms with a choice between artificial diets that contained the same amount of freeze-dried wild parsnip material but different amounts of furanocoumarins.

An artificial diet was prepared according to Nitao and Berenbaum (1988), except that all of the wheat germ and cellulose powder and half of the Wesson's salt mix, sucrose, and vitamin-free casein were replaced with freeze-dried ground powder of wild parsnip buds, flowers, and fruits collected in Champaign County, Illinois, USA, in late May 2001. Half of this diet was further modified by the addition (0.1% fresh wt) of a 94.1% pure mixture of furanocoumarins (containing 1.8% imperatorin, 33.4% xanthotoxin, 53.8% bergapten, 0.51% sphondin, and 4.4% isopimpinellin) isolated from seeds of wild parsnip by the method described in Cianfrogna et al. (2002). This mixture is similar in composition to that of wild parsnip seeds except that the amount of imperatorin is underrepresented. Sixth instars for this experiment were collected from wild parsnip along a railroad track in Kankakee County, Illinois (approximately 127.5 km NE of Champaign, Illinois).

Webworms were transferred to plastic cups, one per cup (29.6 ml capacity; Solo, Urbana, Illinois, USA), containing a preweighed cube of each diet. Once half or more of either diet had been consumed (as judged by eye), the webworms were weighed again, and each diet sample was placed into a new cup and frozen at -20°C. At the end of the experiment, the diet samples were dried in a desiccator and, once the diet samples were at a constant dry weight, all masses were recorded. A preweighed diet cube at the beginning of the experiment was also dried to obtain a wet to dry mass conversion. These values were used to evaluate larval performance on each diet with respect to growth rates, efficiency of utilization of ingested food, as well as diet preference. All larvae readily fed on the plant-containing diet.

To confirm that the furanocoumarin content of diet supplemented with furanocoumarins was higher in furanocoumarin content than the unsupplemented diet, we analyzed content of nine randomly selected samples of each diet type remaining after feeding. Each dried diet sample was ground to a fine powder using a modified Wig-L-Bug (Dental Manufacturing Co., Chicago, Illinois, USA). Furanocoumarins were extracted from the ground diet samples in 0.5 ml of ethyl acetate. After 1 hr, the samples were centrifuged for 5 min, and 0.2 ml of the ethyl acetate layer was removed for analysis by normal-phase high-pressure liquid chromatography (HPLC) (Waters, Milford, Massachusetts, USA, with an Alltech, Deerfield, Illinois, USA, Adsorbosphere column, 150×4.6 mm, 5-μm particle size). Samples (20 μl) were injected by a Waters Intelligent Sample Processor (WISP 710B, Waters). The mobile phase consisted of 55% cyclohexane, 42% isopropyl ether, and 3% butanol flowing at 1.5 ml/min, and detection was at 254 nm.

To determine which of three factors—initial larval mass, amount of control diet consumed, and amount of furanocoumarin diet consumed—influenced final larval mass, we performed multiple regression analysis. To determine whether webworms exhibited a preference between diets, a one-sample *t* test was performed in which the average proportion of diet consumed that contained no furanocoumarins was compared to 0.5, the expected proportion resulting from random choice. All statistical analyses were performed using a SPSS statistical package (SPSS, 1999).

Choice between Filled P. sativa Fruits Treated or Not Treated with Furanocoumarins. In order to determine whether *D. pastinacella* larvae can distinguish among green fruits of *P. sativa* with differing furanocoumarin levels, we presented webworms with fruits to which additional furanocoumarins had been added topically and untreated fruits from the same umbel. Primary umbel fruits of five wild parsnip plants were collected from a roadside population in Kankakee County, Illinois (approximately 127 km NE of Champaign, Illinois). Parsnip fruits are schizocarps, consisting of two mericarps, each of which typically contains a single seed. Parthenocarpic fruits, which contain two empty mericarps, were separated from filled fruits with the aid of an illuminator and reserved with a sample of fully filled fruits for chemical analysis (see below); all half-filled fruits (one mericarp empty) were discarded. Fifty filled fruits from each plant were treated with a furanocoumarin mixture dissolved in acetone (the same mixture of furanocoumarins as

used in the previous experiment), and 50 filled fruits from each plant were treated with acetone alone. Fruits were treated by passing a mixture of furanocoumarins in acetone over them in a Büchner funnel. Solvent was removed by applying suction to the funnel and subjecting the fruits to a constant flow of air for 2 min. To confirm that the fruits had been appropriately supplemented, they were placed under a UV light (long wavelength). Because furanocoumarins fluoresce under UV, the treated fruits fluoresced yellow, whereas the untreated fruits did not. Each fruit was then individually marked with a permanent marker to distinguish between the treated and untreated fruits. A line was drawn on the edge of each untreated fruit starting at the stigma end and extending along both margins halfway to the opposite end of the fruit. For the treated fruits, a line was drawn with a permanent marker on the edge of the fruit starting with the carpophore end and going halfway to the distal end. Once the fruits had been marked, they were thoroughly mixed (100 fruits in all) and placed into Petri dishes (14 cm) with 15 sixth-instar webworms. The experiment was replicated five times, each time with fruits from a different plant. To maintain humidity, we placed a plastic cap containing 1.2 ml of water in each dish. When about half of the fruits had been eaten (approximately 24 hr), the webworms were removed and the uneaten fruits were counted. To determine which fruits the webworms preferred, Pearson chi-square analysis was performed.

To confirm that the supplemental furanocoumarins remained on the fruit for the duration of the experiment, we took four treated and four untreated fruits that had not been eaten from each of the five replicates. The untreated fruits were dipped in acetone to remove any small amounts of furanocoumarins that might have been transferred through webworm feeding and were allowed to air-dry. All fruits were cut widthwise across the vittae and soaked in 0.5 ml of ethyl acetate for 30 min. Samples were centrifuged for 2 min before approximately 0.2 ml of the ethyl acetate was removed from each sample and analyzed by HPLC as described above.

Chemical Differences between Normal (Filled) and Parthenocarpic Fruit. To determine which chemicals might influence webworm preference for parthenocarpic fruits, we analyzed the quantities of 33 constituent chemicals in filled and parthenocarpic fruit from the fruits collected for the previous experiment. Prior to analysis, we confirmed in feeding trials that webworms preferred the parthenocarpic fruits over filled fruits from the same umbel. Three parthenocarpic and three filled fruit samples were analyzed per umbel. Each of the three samples consisted of three fruits, which were broken apart with forceps in 0.3 ml of water before being homogenized with a ground glass tissue grinder inside a 1.5 ml microfuge tube. Once the fruits were homogenized, 0.2 ml of ethyl acetate containing 0.01% hexadecane as an internal standard was added to each tube. Homogenates were thoroughly vortexed before being centrifuged at maximum speed (13,000*g*) for 2 min. One microliter of each extract was analyzed by gas chromatography (Hewlett Packard 5890) for separation and quantification of chemical constituents (Alltech, SE-30, 30-m capillary column, 0.32 mm, ID, 0.25 μm film thickness; temperature program: 60°C, 5 min, 5°/min, 175°C, 2°/min, 200°C, 5°/min, 290°C, 1 min).

Compounds were identified by injecting a representative sample into a Hewlett Packard gas chromatograph—mass spectrophotometer. Additionally, confirmation was made by retention time for compounds with authentic standards (Aldrich, Milwaukee, Wisconsin, USA).

RESULTS

Choice between Plant-Containing Artificial Diet with High or Low in Furanocoumarin Content. HPLC analysis showed that adding furanocoumarins to the artificial diet containing freeze-dried parsnip increased furanocoumarin level threefold over the control artificial diet containing parsnip ($T = 6.94$, $df = 16$, $P = 0.00$). Despite this large difference in furanocoumarin concentration, webworms exhibited no preference ($T = 0.668$, $df = 37$, $P = 0.508$), and the amounts of each diet consumed did not affect larval growth, as evidenced by nearly identical partial regression slopes (0.200 and 0.206, Table 1).

Choice between Filled P. sativa Fruits Treated or Not Treated with Furanocoumarins. On average, augmentation raised furanocoumarin levels in treated fruits by 20% ($T = 2.77$, $df = 4$, $P = 0.025$). Pearson chi-square analysis indicated that parsnip webworms showed a marginal preference for untreated fruits in one of the plants analyzed ($P = 0.046$), a distinct preference for untreated fruits from another plant ($P < 0.001$), and a marginally nonsignificant preference for untreated fruits in one plant ($P = 0.069$). Webworms exhibited no preference in two other plants ($P = 0.546$ and $P = 0.826$). HPLC analysis confirmed that the differences in furanocoumarin content between treated and untreated fruits in the two plants for which no preference was exhibited were similar to those from plants in which a preference was shown.

TABLE 1. MULTIPLE REGRESSION OF RELATIVE CONTRIBUTIONS OF AMOUNT OF ARTIFICIAL PARSNIP DIET CONSUMED WITH OR WITHOUT ADDITIONAL FURANOCOUMARINS TO FINAL LARVAL MASS (DEPENDENT VARIABLE) OF *Depressaria pastinacella*[a]

	Regression Coefficients		Standardized Regression Coefficients		
Independent variables	*B*	SE	β	*t*	*P*
Diet consumed (g), no additional furanocoumarins	0.200	0.075	0.307	2.650	0.012
Diet consumed (g) with additional furanocoumarins	0.206	0.074	0.320	2.776	0.009
Initial larval mass (g)	0.682	0.091	0.777	7.494	<0.001

[a] Regression was significant (ANOVA $F = 20.344$; $df = 3,34$; $P < 0.001$).

TABLE 2. CHEMICAL CONSTITUENTS OF FILLED AND EMPTY *Pastinaca sativa* FRUITS AND CORRELATION WITH FURANOCOUMARINS

Peak	Mean peak area[a] Filled fruits	Mean peak area[a] Empty fruits	*t*	*df*	*P*[c]	Filled empty fruit ratio[1]	Present in all plants	Correlated with furanocs	ID
1	6.42	3.47	2.5	9	0.031	1.9	No	0.494	butyl butyrate[b]
2	2.78	2.08	2.4	9	0.038	1.3	No	0.734	octanol[b]
3	27.78	14.15	4	9	0.003	2	Yes	n.s	hexyl butyrate[b]
4	72.33	42.3	5.7	9	<0.001	1.7	Yes	0.616	decanal
5	217.5	95.91	2.6	9	0.028	2.3	Yes	n.s	octyl acetate[b]
6	35.11	15.89	5.4	9	<0.001	2.2	Yes	0.728	
7	2514.9	1361.3	7.5	9	<0.001	1.8	Yes	0.953	octyl butyrate[b]
8	3.29	1.24	1.4	9	0.181	2.7	No	0.544	bergamotene
9	2.4	1.52	1.2	9	0.253	1.6	No	n.s.	2-methyl-2-hexene
10	8.28	6.29	5	9	0.001	1.3	Yes	0.759	
11	56.12	41.19	4.9	9	0.001	1.4	Yes	0.790	myristicin[b]
12	240.8	119.2	5.7	9	<0.001	2	Yes	0.886	
13	12.83	8.48	6.5	9	<0.001	1.5	Yes	0.789	
14	7.02	6.55	1.6	9	0.145		Yes	0.577	
16	26.25	6.55	1.7	9	0.133		No		angelicin[b]
17	214.6	177.5	0.9	9	0.373		Yes		xanthotoxin[b]
18	114.5	83.22	0.5	9	0.611		Yes	n.s.	
19	129.6	82.71	0.6	9	0.534		No	0.468	
20	274.4	79.1	1.9	9	0.092		No	0.817	
21	328.5	117.6	3.7	9	0.005	2.8	Yes		sphondin[b]
22	65.81	37.68	5.8	9	<0.001	1.7	Yes	0.851	
23	54.48	26.27	2.2	9	0.055	2.1	Yes	n.s.	osthole[b]
24	95.15	48.18	4.8	9	0.001	2:1	Yes		isopimpinellin[b]
25	114.3	72.74	1.4	9	0.183		Yes	n.s.	
26	190.4	81.71	2.8	9	0.022	2.3	No		imperatorin[b]
27	114.3	56.85	3.6	9	0.006	2	Yes	0.846	
28	27.83	8.22	2.6	9	0.03	3.4	No	0.909	
29	11.42	4.22	2.1	9	0.069		No	0.762	
30	24.97	16.24	6.1	9	<0.001	1.5	Yes	0.571	
31	13.16	8.09	3	9	0.014	1.6	Yes	0.570	
32	13.43	6.98	2.6	9	0.03	1.9	No	0.710	
33	30.42	21.14	3.1	9	0.013	1.4	Yes	0.490	

[a] Peaks are in integrator units with each unit having a value of 0.125 μV-sec.
[b] Identified both by mass spectrum and retention time against standard.
[c] Given only for compounds that differed significantly between fruit types based on paired *t* test. n.s. Not significant.

Chemical Differences between Normal (Filled) and Parthenocarpic Fruit. A significant difference in peak area between filled and empty fruits was found for 21 of the 33 peaks recorded. Of the 33 peaks, 21 were present in all plants assayed and 19 were positively correlated with furanocoumarins (Table 2). Of

the 12 constituents that were different between fruit types, present in all plants, and correlated with furanocoumarin content, we identified three as octyl butyrate, myristicin, and decanal. The correlations between total furanocoumarin content and octyl butyrate and myristicin content were significant and positive (0.953 and 0.790, respectively; Figure 1), whereas the correlation involving decanal was considerably lower (0.616).

DISCUSSION

The parsnip webworm does not sequester furanocoumarins in its body for defense against predators; rather, it relies on rapid cytochrome P-450-mediated metabolism of furanocoumarins (Berenbaum and Zangerl, 1992). Ever since Verschaffelt's (1910) demonstration that *Pieris* spp. consume nonhost tissues treated with sinigrin, a glucosinolate component of their cruciferous host plants, there has been an expectation that highly oligophagous insects detect and recognize host plants by virtue of their distinctive chemistry (Städler, 1992). Demonstrations of such unequivocal responses to diagnostic host chemicals, however, are less frequent than might be expected. Although species that sequester host-plant chemicals for defense often display distinct feeding preferences for those chemicals, not all sequestering species are immune to the negative effects of these chemicals. Young monarch butterfly larvae, for example, experience reduced growth and survival associated with feeding on latex and cardenolides in *Asclepias humistrata* (Zalucki et al., 2001). Oligophages that do not sequester chemicals to which they are presumably well adapted may nevertheless be adversely affected by high concentrations (Siemens and Mitchell-Olds, 1996) and might be expected to avoid high concentrations.

When, in the first choice experiment, webworms were presented with a threefold difference in furanocoumarin content of artificial diets containing host-plant tissue, they exhibited no preference. This lack of preference is consistent with the only other study of furanocoumarin feeding preference by webworms (Berenbaum and Zangerl, 1992). However, the experiment reported here more closely resembles the situation confronting webworms in nature because more of the furanocoumarins commonly found in green wild parsnip tissues were assayed [five of seven rather than three of seven in Berenbaum and Zangerl (1992)], and the assay was performed in the context of other wild parsnip chemicals. More realistic yet is the choice test involving whole green fruits with and without supplemental furanocoumarins. The results of this experiment suggest that webworms are generally not able to detect differences in the levels of furanocoumarins and behave in a way that minimizes their exposure to these toxins. Instead, detection of furanocoumarins, if it occurs at all, appears to depend on the fruit characteristics peculiar to the plants from which they are taken. Shields and Mitchell

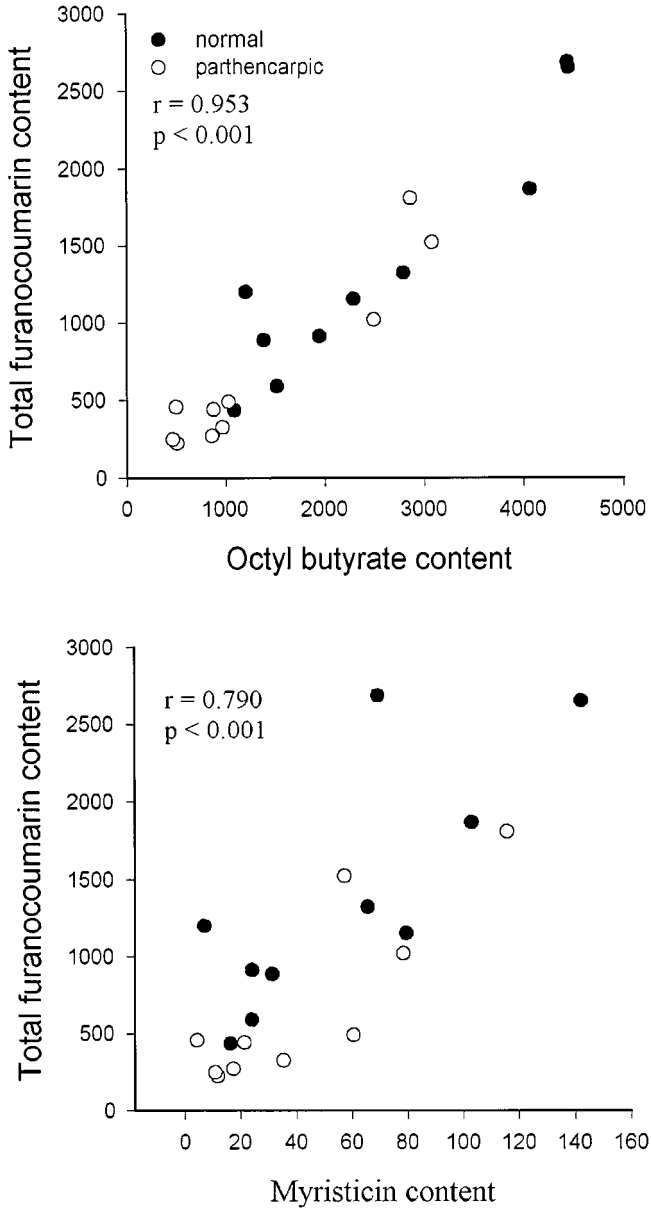


FIG. 1. Correlations between total furanocoumarin content and octyl butyrate and myristicin in filled and parthenocarpic fruits of wild parsnip. Contents are expressed as areas in integrator units.

(1995) similarly reported that the deterrent properties of sinigrin, a glucosinolate, to crucifer-feeding caterpillars are dependent upon the presence or absence of stimulatory sugars and sugar alcohols.

Although webworms exhibit a distinct preference for parthenocarpic fruit (Zangerl et al., 1991), which are lower in furanocoumarins, it appears unlikely from the choice experiments in this study and in an earlier one (Berenbaum and Zangerl, 1992) that furanocoumarins are independently assessed and avoided. If other compounds can be detected that are highly correlated with furanocoumarins, webworms may use them as cues to assess furanocoumarin levels. Such compounds would be expected to differ between parthenocarpic and normal fruit, and, presumably, should be present in all parsnip plants. Using these criteria, we narrowed the number of candidate chemical constituents detected by gas chromatography from 33 to 12, of which three have been positively identified. Of these, octyl butyrate is known to be a feeding deterrent for the parsnip webworm (Carroll and Berenbaum, in press). The high correlation ($r = 0.953$) between furanocoumarin content and octyl butyrate content detected in this study suggests that webworms could precisely assess furanocoumarin content of fruits indirectly by assessing octyl butyrate content. Another possible candidate that may explain feeding preference is myristicin, which is also correlated with furanocoumarin content and may be harmful by inhibiting activity of the cytochrome P-450s involved in metabolism of furanocoumarins (Berenbaum and Neal, 1985). Other chemical constituents that were not quantified or those such as decanal or the other nine unidentified constituents might be responsible for the feeding behavior in parsnip webworm, but further work is needed to make such a determination.

This study suggests that feeding behaviors of insect herbivores, including those with a restricted host range, may not be guided directly by those plant constituents that pose a potential risk for the insect. The level of host-plant toxins indicated by the level other detectable host-plant constituents may allow webworms to feed on the most suitable plants or plant parts. That webworms cannot consistently avoid higher furanocoumarin content in the presence of other plant constituents but can avoid them in intact plants suggests that the covariance of furanocoumarins with other plant constituents may ultimately govern feeding choice. To the extent that this phenomenon occurs in other herbivorous insects, choice studies, even those involving spiked plant parts, may lead to erroneous conclusions because they do not preserve the correlations between the chemical of interest and other plant constituents that exist in plants and affect feeding behavior.

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EXPERIMENTAL STUDIES OF THE HOST-FINDING BEHAVIOR OF *Trogus pennator*, A PARASITOID OF SWALLOWTAIL BUTTERFLIES

KAREN R. SIME*

Department of Entomology
Department of Ecology and Evolutionary Biology
Cornell University
Ithaca, New York

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Abstract—The parasitic wasp *Trogus pennator* (Hymenoptera: Ichneumonidae) attacks larvae in two genera of Papilionidae, *Eurytides* and *Papilio*, on plants in a variety of families. The female wasps' responses to food plants, feeding damage, and frass were examined in a series of experiments designed to test the hypothesis that parasitic wasps that specialize on host taxa and seek their hosts in a variety of habitats exhibit fixed responses to host-derived cues and more flexible responses to cues associated only with the hosts' food plants. Naive *T. pennator* females showed no preferences when offered either a choice between two papilionid food plants or a choice between a food plant and a plant not used for food by Papilionidae. After experience with hosts in the presence of a particular food plant, however, wasps preferred that plant. Naive wasps did prefer plants damaged by host larvae over plants damaged by nonhost (saturniid) larvae and also preferred methylene chloride extracts of host frass over extracts of frass from saturniid larvae fed on the same plant species, results indicating that the responses of *T. pennator* females to host-derived cues are innate. The chemical compositions of the extracts of frass from several papilionid and one saturniid species were also examined, and the significance of the finding that no host-specific patterns were detected among the major components of the extracts is discussed.

Key Words—Ichneumonidae, Papilionidae, host-finding behavior, learning, feeding damage, semiochemicals, frass, flight chamber.

* E-mail: krs1@cornell.edu

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INTRODUCTION

Parasitic wasps in the genus *Trogus* Panzer (Ichneumonidae) lay their eggs in the larvae of swallowtail butterflies (Lepidoptera: Papilionidae) and emerge as adults from the remains of the host pupae (Mitchell, 1979, 1981). The North American species *T. pennator* (Fabricius) is widespread in the United States, although it is most common in the southeastern states, and is relatively polyphagous, attacking nearly all the papilionids in its range, including *Eurytides marcellus* (Cramer) and at least five *Papilio* species (Heinrich, 1962; Mitchell, 1983; Sime and Wahl, 2002). These hosts feed on a variety of plants, although with the exception of the generalist *P. glaucus* L., each species tends to specialize within plant families (Feeny, 1991). *Trogus pennator* has been reared from larvae feeding on eight plant families, suggesting that it does not discriminate among its hosts' food plants (Sime, 2001).

The host-finding behavior of *T. pennator* has been studied under natural conditions in the Ocala National Forest, Florida, USA, where the usual host is the zebra swallowtail, *E. marcellus* (Sime, 2001), which feeds on species of pawpaw (Annonaceae: *Asimina*). By following females as they searched for host larvae, it was established that the wasps discriminate between *Asimina* and other plants, that they are attracted to plants damaged by hosts, and that they spend more time searching on damaged than on intact plants. While that study provided valuable information about the behavior of undisturbed wasps in a natural environment, it left several important questions unanswered. The evidence indicated that the wasps rely at least in part on vision to identify *Asimina* plants, but it was not possible to determine whether they also respond to plant odor, as do most parasitic wasps (Vinson, 1984). It was also not possible to establish whether the wasps recognize feeding damage on the basis of odor, or whether they can distinguish the feeding damage of their hosts from that left by other lepidopteran larvae.

To discover the nature of the cues involved in orienting to plants, laboratory experiments were conducted to determine whether wasps are attracted to plant odor and whether the sources of useful odors include intact plants as well as plants chewed by host larvae. To establish a chemical basis for their arrestment behavior, the responses of wasps to chemical extracts of host frass were examined and an attempt was made to isolate and identify active compounds.

Another shortcoming of the previous field study was that it was not possible to know whether the observed attraction to plants had been learned, as is the case for many parasitic wasps (Vet et al., 1995). No information was available on the life experiences of the wasps observed, although presumably all had encountered hosts, which were abundant at the site. Because the host range of *T. pennator* and the food plants of its hosts are well known, an investigation of learning and cue specificity in this species provides an opportunity to test ideas concerning the evolution of host range in parasitoids. Gauld (1988) and Vet and Dicke (1992)

have hypothesized that for parasitoids specializing on particular host taxa, host range is a function of the distribution of chemical compounds used to find and identify hosts. This hypothesis requires, first, that innately attractive cues exist (i.e., the wasps are attracted to them regardless of experience and environment), and second, that the parasitoids are able to use these cues to discriminate hosts from nonhosts. For *T. pennator*, this hypothesis predicts that the females use compounds characteristic of papilionid larvae to find and recognize these hosts. Responses to hosts are expected to be fixed, but responses to plant-derived cues may be more flexible. This hypothesis was tested in the present study by investigating whether plant- and host-derived chemical cues were innately attractive or learned and by comparing responses to odors produced by papilionid and non-host larvae.

METHODS AND MATERIALS

Sources of Insects and Plants. Most of the *Trogus pennator* females used in these experiments were the offspring of adults caught in the Ocala National Forest, Florida, in late March and early April of 1995, 1997, and 1998. A few were netted at patches of *Asimina triloba* (L.) (a food plant of *E. marcellus*) along the James River near Buchanan, Virginia, USA, in July of 1996 and 1998. The wasps were raised on larvae of *E. marcellus*, also collected in Florida, that had been exposed to female wasps in captivity and maintained on cuttings of *A. speciosa* Nash and *A. triloba*. Adult wasps were kept for up to several weeks at room temperature with unlimited access to water and a honey-water solution. They were refrigerated (at 4°C) for up to a week at a time when not needed for experiments. Females were intermittently allowed to attack *E. marcellus* larvae as necessary to maintain the colony.

Several other species of Papilionidae were reared for use in experiments. Larvae of *Papilio troilus* L., taken from a laboratory colony maintained on the Cornell campus (Carter et al., 1999), were reared on foliage clipped from sassafras trees [*Sassafras albidum* (Nutt.)] in Ithaca, New York, USA. Larvae of *Papilio polyxenes* Fabricius, also taken from a local colony, were reared on leaves from potted carrot plants (*Daucus carota* L.) (Brooks, 2000). The offspring of *Papilio glaucus* L. females caught in the Ithaca area were reared on ash (*Fraxinus americana* L.). In addition, a nonpapilionid species, *Callosamia promethea* (Drury) (Saturniidae), was reared for use as a control. This species was chosen because it is readily available and because, like *P. troilus* and occasionally *P. glaucus*, it feeds on sassafras, thus introducing a control for the effects of food plant on host odors. The *C. promethea* larvae were the offspring of moths obtained as pupae from an amateur supplier in New Jersey and were reared on the leaves of local sassafras trees under the same conditions used to rear *P. troilus*.

All insects were bred and reared in a climate-controlled chamber (16L:8D, 30/16°C day/night, and 80–85% relative humidity). Wasps used in experiments

requiring naive females had no contact with hosts or with any plant matter between the time of their emergence and the time of the experiment. The pupae from which they emerged had been cleaned of silk and plant residue and placed on clean paper towels in a nylon-screened cage.

Voucher specimens of butterflies, moths, and wasps were deposited in the Cornell University Insect Collection (lot no. 1240).

Responses to Plants and Feeding Damage: Flight-Chamber Experiments. The flight chamber was a wooden cage screened with nylon mesh (185 × 92 × 92 cm). Plant samples were placed at one end and separated from each other by a cardboard partition projecting 50 cm into the cage. The wasps were released from vials at the opposite end of the chamber. A 50-cm-diam. fan was placed just outside the plant end of the chamber. Wind speeds were maintained at 1.0 m/sec at the plants, grading down to 0.5 m/sec at the release site, as measured by a handheld anemometer (Davis Instruments, Hayward, California, USA). The assembly was housed in a rooftop greenhouse on the Cornell University campus that was maintained at 24°C. Experiments were conducted near midday on sunny days in midsummer, using the sun as the main light source. The plants were placed in the southern end of the flight chamber to take advantage of the wasps' phototactic responses.

Twigs were cut shortly before each trial (except when exposed to host larvae as described below) and were kept in small vials of water during the trials. The positions of the two cuttings were switched with every fifth wasp. A wasp that did not cross into the partitioned area within 10 min was considered nonresponsive and excluded from the data set. The numbers of wasps that chose each plant species or treatment were compared with binomial probability functions (Conover, 1999).

The first two flight-chamber experiments (1a and 1b) tested whether the wasps learn the odor of swallowtail food plants by comparing the responses of naive and experienced females to a choice of sassafras (the food plant of a host, *P. troilus*) and maple (*Acer platanoides* L.). Maple was chosen as the alternate because it is not fed on by any swallowtails, because it is a common tree in swallowtail habitats in the eastern United States, and because its lobed leaves resemble those of sassafras in gross outline, thus providing a control for visual preferences. Discrimination by naive females would indicate an innate preference. Alternatively, if only wasps with experience attacking hosts in the presence of sassafras favored the food plant, then a learned response would be indicated.

In experiment 1a, females were tested before and after they had experience attacking hosts. The wasps were reared from *E. marcellus* larvae collected in Florida in spring 1996. The naive trials were conducted with 2-week-old mated wasps between June 30 and July 2. After the last trial, the wasps were placed in cages containing *P. troilus* larvae feeding on sassafras, which they attacked immediately. The experienced trials were conducted July 3–6.

Experiment 1b controlled for the age of the wasps. The wasps had been reared from *E. marcellus* larvae collected in Florida in spring 1997. When they

were 2 weeks old, the females were divided into two sets, one of which stayed naive while the other—the experienced set—was placed for a few hours on June 27 and 28 in boxes with first- and second-instar *P. troilus* feeding on sassafras cuttings. The two groups of wasps were tested concurrently from June 29 to July 2.

Experiment 2 tested for an innate preference among different swallowtail food plants. Week-old, mated, naive females were offered a choice of sassafras and *Asimina triloba*, the food plants of *P. troilus* and *E. marcellus*, respectively, the most common hosts of *T. pennator* in the southeastern United States. The wasps were reared from *E. marcellus* larvae collected in Florida in spring 1997 and raised on *Asimina*. Any food-plant effects carrying over from the hosts or pupae were, thus, expected to bias the experiment towards *Asimina*.

Experiment 3 tested whether host damage is innately attractive by examining the responses of naive wasps to a choice of sassafras leaves chewed by *P. troilus* larvae and sassafras leaves that had never been exposed to caterpillars. The naive wasps used in experiment 2 were refrigerated for four days and used again in this experiment. The unexposed sassafras leaves were mechanically damaged with scissors to control for effects of damage unrelated to larval feeding. The "chewed" leaves were exposed to *P. troilus* larvae overnight; the larvae were removed from the leaves 10 min before the start of the experiment.

Experiment 4 expanded on experiment 3 by testing whether the wasps are more attracted to the feeding damage of their hosts than to the damage of other lepidopteran larvae. Naive wasps were offered a choice between sassafras leaves chewed by *P. troilus* and sassafras leaves chewed by the saturniid *C. promethea*. As in experiment 3, the larvae fed on the leaves overnight and were removed from them 10 min before the start of the experiment. Fourth and fifth instars of both species were used, so the amount of damage inflicted on the leaves was similar in the two treatments. The female wasps were reared from *E. marcellus* larvae collected in Florida in spring 1998; they emerged over several days in mid-June and were tested 10 days after emergence.

Responses to Frass Extracts. Methylene chloride extracts of frass were prepared from the following species: *E. marcellus* (35 g frass collected), *P. troilus* (49 g), *P. glaucus* (7 g), *P. polyxenes* (5 g), and *C. promethea* (60 g). The larvae were reared on plant cuttings inserted into plastic vials and placed in plastic boxes. Each morning, frass that had accumulated in the bottom of the box was transferred to a container of CH_2Cl_2 and ground with a glass rod to increase the efficiency of extraction. The extracts were refrigerated at 0°C. After collection was complete (usually after several days), the extracts were filtered under low vacuum through glass wool and Whatman No. 1 filter paper. They were then evaporated under reduced pressure at 40°C to a concentration of 0.25 g frass equivalent/ml.

To test for arrestment responses to these extracts, the time females spent searching in the vicinity of the extract was measured. The extract was presented on a cotton swab in the center of a Petri dish lid (9 cm diam.), supported by a small

piece of green plastic sponge (2 cm square × 0.5 cm high). The swab contained 2–3 drops of extract, which at the given concentration was roughly equal to the amount of frass produced by a fourth- or fifth-instar caterpillar in a few hours of feeding. The Petri lid was centered on the floor of a plastic box (35 × 27 × 60 cm), and the wasps were released into the box from a glass jar placed at one end of the box. Searching time was recorded as the time spent actively antennating the substrate while within the lid; time spent grooming or resting was excluded. Both experiments were carried out in the climate chamber described above.

The first experiment compared the time that naive wasps spent searching at extracts from *P. troilus* and from the nonhost *C. promethea*. The trials were paired such that each wasp spent 5 min with one extract and then 5 min with the other (the order of presentation of extracts varied randomly from wasp to wasp). The average times spent at the extracts were compared with a two-tailed paired *t* test. The wasps originated in Florida and had been reared from *E. marcellus* larvae collected in spring 1998.

To get a better idea of the extent to which these responses were particular to swallowtail frass, a second experiment was conducted which tested the wasps' responses to extracts from the three *Papilio* species (*P. troilus*, *P. polyxenes*, and *P. glaucus*) and the *C. promethea* control. Each wasp spent 4 min with each of the extracts in succession, the order of presentation being varied at random. The wasps used in this experiment had been caught in Virginia at *Asimina* patches and were assumed to have had experience in the field with *E. marcellus* and possibly *P. troilus*. Because the wasps switch readily between host species in the laboratory (K.R.S., personal observation), such experience was not expected to affect the outcome of the experiment.

Characterization of Frass Extracts. The preceding experiments compared the arrestant activities of the *C. promethea*, *P. troilus*, *P. polyxenes*, and *P. glaucus* frass extracts. The *E. marcellus* extract was simply tested against the solvent with a bioassay identical in design to the paired experiment described above. Following the biosassays, the compositions of the papilionid and nonpapilionid extracts were characterized and compared.

Each extract was fractionated on an open column containing 30 g silica gel (Si-60, 32–63 μm). Five fractions were collected, corresponding to five additions of 250 ml solvent: pentane, pentane–methylene chloride (1:1), methylene chloride, methylene chloride–ether (1:1), and ether. The *P. troilus* extract was analyzed in detail. Each of its five fractions was bioassayed in paired experiments like those described above, comparing the searching activities elicited by the fraction and a solvent control. When the third fraction was identified as active, it was tested against a recombination of the five fractions (controlling for concentration) in order to determine the extent to which activity was limited to that fraction. Fractions of the *E. marcellus*, *P. glaucus*, *B. philenor*, and *C. promethea* extracts comparable to the third fraction of the *P. troilus* extract were prepared using the same procedures

to fractionate the original CH_2Cl_2 extracts. The eluting samples were monitored with thin-layer chromatography using a solvent system of equal portions hexane and ethyl acetate.

These fractions were analyzed using a Hewlett-Packard HP5890 gas chromatograph coupled to an HP 5970B mass-selective detector. The GC employed helium as carrier gas, with a flow rate of 1 ml/min, and was fitted with an HP-1 capillary column. Injections were splitless. The temperature program started at 60°C, rising at a rate of 10°/min to 260°C, then holding for 5 min at 260°C. The major components of the samples were identified by matching EI spectra recorded at 70 eV to spectra in the Wiley library.

Finally, to facilitate identification of active compounds, the active fraction of the *P. troilus* extract was further split into three subfractions (A, B, C). The fractionation procedures were like those described above except that the solvent system consisted of successive additions of 250 ml hexane–chloroform (1:4), chloroform, and chloroform–ethyl acetate (1:1). Each of the three resulting subfractions was bioassayed against chloroform.

RESULTS

Responses to Plants and Feeding Damage: Flight-Chamber Experiments. The outcomes of experiments 1a and 1b were similar (Table 1), with the less conclusive results of 1a possibly indicating effects of age. Naive females did not discriminate between maple and sassafras leaves, but the wasps that had attacked *P. troilus* larvae feeding on sassafras tended to fly towards sassafras. These results

TABLE 1. RESPONSES OF NAIVE AND EXPERIENCED WASPS TO SASSAFRAS AND MAPLE (*Acer*) LEAVES IN A FLIGHT CHAMBER[a]

	Wasps favoring		
	Sassafras	Maple	*P*
Experiment 1a			
Naive	11	13	0.42
Experienced	14	7	0.09
Experiment 1b			
Naive	16	19	0.37
Experienced	26	14	0.04

[a] In Experiment 1a, wasps were tested before and after experience with hosts feeding on sassafras. In Experiment 1b, two sets of wasps the same age were tested, one of which was given such experience. Significance levels are based on binomial probability functions.

TABLE 2. EXPERIMENTS 2, 3, AND 4: RESPONSES OF NAÏVE WASPS TO VARIOUS CHOICES OF PLANT TREATMENTS IN A FLIGHT CHAMBER

	Plant choices		*P*
Experiment 2	Sassafras leaves	*Asimina* leaves	
Wasps favoring each treatment (*N*)	12	14	0.42
Experiment 3	Sassafras damaged by *P. troilus*	Artificially damaged Sassafras	
Wasps favoring each treatment (*N*)	20	10	0.05
Experiment 4	Sassafras damaged by *P. troilus*	Sassafras damaged by *C. promethea*	
Wasps favoring each treatment (*N*)	14	6	0.05

indicate that responses to plants *not* damaged by hosts are learned. Assuming that differences in visual cues were minimized between the two treatments, these experiments also show that odor plays a role in plant recognition. Because color and shape cues in the leaves were not strictly controlled for, however, the possibility that visual cues contributed to the wasps' responses cannot entirely be eliminated.

Naive wasps did not discriminate between two common swallowtail food plants, *Asimina* and sassafras (Table 2, experiment 2). Because the wasps used had been reared from *E. marcellus* larvae feeding on *Asimina*, this experiment also shows that neither the host nor its food plant biases the preferences of a naive wasp.

Experiment 3 indicated that naive wasps favor leaves damaged by hosts over mechanically damaged leaves, while experiment 4 showed that naive wasps prefer leaves damaged by a host over leaves damaged by another lepidopteran species (Table 2). Since there were no differences in the appearances of the leaves used in these treatments, it may be concluded that the wasps were responding to odors associated with host damage.

Responses to Frass Extracts. Naive wasps searched longer at a methylene chloride extract of the frass of *P. troilus* (a host) than at an extract from *C. promethea* (a nonhost) (Figure 1). Arrestment responses to extracts of the frass of *P. troilus*, *P. glaucus*, and *P. polyxenes* were similar, and all were significantly greater than responses to the extract from *C. promethea* (Figure 2). Because both the *P. troilus* and the *C. promethea* larvae had fed on sassafras, both experiments show that the wasps respond to odors in frass that are peculiar to host larvae.

Characterization of Frass Extracts. The preceding experiments showed that the methylene chloride extracts of the frass of *P. troilus*, *P. glaucus*, and *P. polyxenes* larvae elicit an arrestant response in *T. pennator*. Similarly, an extract from *E. marcellus* was searched significantly longer than a solvent control (Table 3). Bioassays of the five fractions of the *P. troilus* extract indicated that

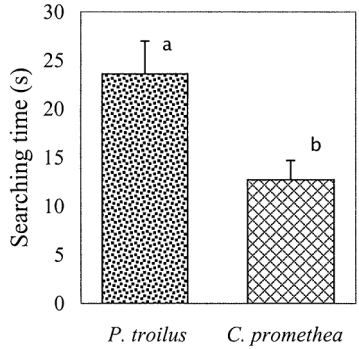


FIG. 1. Mean searching times of naive female wasps at methylene chloride extracts of frass from a papilionid host, *P. troilus*, and from the saturniid moth *C. promethea* ($N = 17$, $P < 0.01$). Both species were reared on sassafras foliage.

nearly all of the arrestant activity could be attributed to fraction 3 (Table 3): the wasps searched significantly longer at fraction 3 than at the solvent control, and its activity was comparable to that of the recombined fractions. Of the three subfractions prepared from fraction 3, only the middle one (3B) elicited greater activity than the solvent control.

A number of compounds were identified in fraction 3 of frass extracts from *C. promethea*, *P. troilus*, *E. marcellus*, and *P. glaucus* (Table 4). Concentrations of compounds in fraction 3B of *P. troilus* proved insufficient to make reliable identifications.

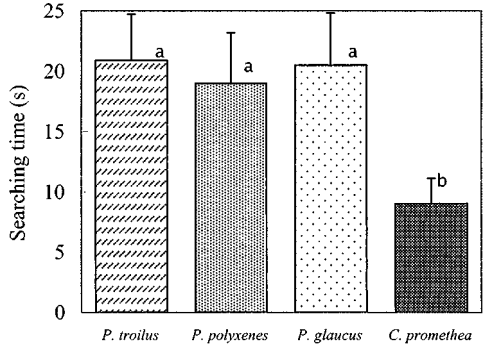


FIG. 2. Mean searching times of female wasps at methylene chloride extracts of frass from three host *Papilio* species and the saturniid *C. promethea* ($N = 24$, $P < 0.05$).

TABLE 3. BIOASSAYS OF METHYLENE CHLORIDE EXTRACTS OF FRASS AND FRACTIONS THEREOF[a]

Treatment 1	Treatment 2	*N*	*P*
E. marcellus extract	solvent	11	
$41.2 \pm 9.6^*$	18.3 ± 3.3		0.027
P. troilus fraction 1	solvent	16	
26.9 ± 7.4	14.5 ± 5.6		0.15
P. troilus fraction 2	solvent	8	
18.3 ± 8.0	4.2 ± 2.0		0.14
P. troilus fraction 3	solvent	11	
55.3 ± 17.0	28.4 ± 12.9		0.044
P. troilus fraction 4	solvent	10	
13.3 ± 5.0	4.81 ± 3.2		0.26
P. troilus fraction 5	solvent	8	
4.9 ± 5.0	2.86 ± 1.0		0.33
P. troilus recombined fractions 1–5	*P. troilus* fraction 3	14	
41.6 ± 6.0	35.6 ± 7.4		0.47
P. troilus fraction 3A	solvent	15	
22.5 ± 5.5	21.8 ± 5.6		0.91
P. troilus fraction 3B	solvent	15	
23.3 ± 5.5	6.2 ± 1.8		0.0047
P. troilus fraction 3C	solvent	15	
13.8 ± 4.1	8.7 ± 3.6		0.16

[a] Mean searching times (seconds) ±SE are presented for each treatment; the means were compared by two-tailed paired *t* tests.

DISCUSSION

The flight-chamber experiments, although limited in the number of plant species tested, provide evidence that *T. pennator* females emerge without preference for the food plants of swallowtails over other plant species or for particular swallowtail food plants over others. The Ocala National forest is at the southern edge of the geographical range of sassafras (Brown and Kirkman, 1990). The wasps used in these experiments therefore came from a population whose members do not normally encounter sassafras, although they may encounter *P. troilus* larvae feeding on another lauraceous plant, *Persea borbonia* L. (Lederhouse et al., 1992). That they can learn to associate the odor of sassafras with the presence of host larvae indicates considerable flexibility in their capacity to recognize plants, as would be expected in a widespread species that attacks hosts feeding on a diverse array of plant families (Vet and Dicke, 1992). The attraction to undamaged plants observed in the flight chamber also helps explain the observation that in the field, female *T. pennator* approach and search food plants lacking feeding damage significantly more often than they approach damaged plants (by a ratio of about 2:1) (Sime, 2001). While the field observations suggested that vision plays

TABLE 4. METHYLENE CHLORIDE EXTRACTS OF FRASS COMPARED VIA GC-MS[a]

	C. promethea	*P. troilus*	*P. glaucus*	*E. marcellus*
Long-chain acids				
Tetradecanoic acid (16.8)*				+
Pentadecanoic acid (17.9)				+
Hexadecanoic acid (18.5)	+		+	+
Octadecanoic acid (21.0)				+
Fatty acid esters				
9,12,15-Octadecatrienoic acid, methyl ester, (*Z,Z,Z*) (20.3)	+	+	+	+
9,12-Octadecadienoic acid (*Z,Z*) methyl ester (20.8)				+
Hexanedioic acid, dioctyl ester (22.5)			+	+
Terpenoids				
β-Citronellol (9.3)	+			+
trans-Geraniol (9.7)	+			+
Neophytadiene (17.4)	+	+		+
(−) - loliolide (15.9)			+	
Long-chain alcohol				
9,12,15-Octadecatrien-1-ol (12.1)	+			+
Long-chain aldehyde				
9,12,15-Octadecatrienal (20.2)	+			
Aromatic aldehydes				
^{1}H-Indole-3-carboxaldehyde (16.7)				+
4-Hydroxy-3-methoxy-benzeldehyde (11.9)				+
Aromatic esters				
1,2-Benzenedicarboxylic acid, bis (2-ethylhexyl) ester (24.7)				+
Aromatic acid				
benzoic acid (8.7)				+
Benzofuranone, benzopyranone				
5,6,7,7a-Tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone (13.7)				+
7-Hydroxy-6-methoxy-2H-1-benzopyran-2-one (18.5)		+		

[a] The compounds listed were identified with a relatively high degree of certainty (>90%) based on library matches. Retention times are given in minutes. '+' = present.

a role in plant recognition, the flight-chamber experiments show that the odor of undamaged plants can also be learned.

One consequence of the attraction of naive wasps to odors associated with feeding damage is that they need not land on and search plants at random before their first encounters with host larvae, but instead can rapidly come to focus on the host's food plant. Field data for female *T. pennator* indicate that the feeding damage of *E. marcellus* larvae on *Asimina* plants is detected at distances of at least 1 m (Sime, 2001); studies of other species, conducted in flight chambers or

wind tunnels, have also provided evidence that feeding-damage odor is involved in medium-range (on the order of meters) orientation (e.g., Geervliet et al., 1994). The attraction of naive wasps to the so-called plant-host odor complex has been observed in many species of Braconidae (Turlings et al., 1990; Steinberg et al., 1992; Udayagiri and Jones, 1993; Reed et al., 1995; Geervliet et al., 1996) and Ichneumonidae (Lecomte and Thibout, 1986; Vinson and Williams, 1991). Other studies have found that the odor of lepidopteran feeding damage is species specific and that parasitoids can distinguish host odors from those of nonhosts (Blaakmeer et al., 1994; Agelopoulos et al., 1995; DeMoraes and Mescher, 1999), although such discrimination is not always observed (McCall et al., 1993; Geervliet et al., 1996).

It has also often been shown that naive wasps are attracted to the frass of their hosts (Lewis and Jones, 1971; Hendry et al., 1973; McKinney and Pass, 1977; Lewis and Tumlinson, 1988; Vet and Dicke, 1992; Suiter et al., 1996). It is usually assumed that the wasps are able to distinguish the frass of their hosts from that of other species, but most studies of frass do not test responses to nonhosts or distinguish compounds of plant origin from those modified by digestion and metabolism in the larvae (Ramachandran et al., 1991). The finding that *T. pennator* females favored *P. troilus* frass over *C. promethea* frass when both species were reared on sassafras indicates that the frass of two species feeding on the same food plant can differ in odor and that the wasps recognize the difference. Furthermore, swallowtail frass is recognized and about equally attractive regardless of food plant. In the four-way experiment, the *Papilio* species had fed on Lauraceae (*P. troilus*), Apiaceae (*P. polyxenes*), and Oleaceae (*P. glaucus*). To this list may be added Aristolochiaceae: a separate study showed that a methylene chloride extract of the frass of *Battus philenor* (L.) larvae feeding on *Aristolochia* was as attractive to *T. pennator* females as was an extract from *P. troilus* (Sime, 2002). Whereas the Lauraceae and Apiaceae resemble each other in odor and essential oil content, and the Aristolochiaceae share some of these chemical qualities, the Oleaceae are chemically very different (Feeny, 1991).

The chemical basis of frass discrimination is difficult to pinpoint. None of the compounds or classes of compounds identified in the active fraction of the frass extracts (Table 4) is characteristic of swallowtails, and there appears to be as much overlap in frass composition between *C. promethea* and any given swallowtail as there is among swallowtail species. Even if any of these compounds should prove attractive to *T. pennator*, it is unlikely that any one compound would provide a basis for distinguishing host from nonhost. Two bases for host discrimination are possible, and neither can be rejected with the available evidence. Critical compounds that characterize swallowtails might be among the minor, unidentified constituents of the extracts. Alternatively, the wasps may respond not to the presence or absence of particular compounds, but instead to a particular blend of compounds; such blends have been found to be critical for recognition of host odors associated with feeding

damage (Turlings et al., 1990, 1991; Tumlinson et al., 1992, 1993; Agelopoulos and Keller, 1994; Geervliet et al., 1994). The observation that the wasps searched at the *C. promethea* extracts at all merits mention, for it indicates that, despite their evident preference for swallowtail frass, the wasps may also have generalized responses to the odors of frass. The compounds eliciting the arrestment response would, thus, appear to include a combination of swallowtail-specific cues plus more general cues of either caterpillar or plant origin. Finally, the possibility that *C. promethea* frass contains deterrent compounds should not be dismissed, as it was not accounted for in the design of these experiments.

These results suggest a general caveat for the interpretation of data concerning the semiochemical content of frass. Although many evidently attractive compounds have been identified in the frass of lepidopteran larvae (Tumlinson et al., 1992; Rutledge, 1996), the ecological roles of these compounds have not always been clearly demonstrated. It is usually not known whether any are individually characteristic of the host or if they are simply components of attractive blends used to identify hosts. The distribution of identified attractant compounds within Lepidoptera is not known; in many systems it would be useful to know which appear in the frass of other lepidopteran larvae feeding on the same or similar plants. Compounds widespread in Lepidoptera cannot by themselves permit host recognition.

Although the active compounds or blends that permit *T. pennator* females to distinguish papilionid larvae from nonhosts were not identified, the work reported here provides evidence that hosts differ chemically from nonhosts and that this difference is discerned by wasps with no prior experience of hosts. Odors associated with both host-damaged leaves and host frass were distinguished from odors produced by a saturniid feeding on the same plants. These findings indicate that the host range of *T. pennator* is a function of attractive compounds shared by and unique to swallowtail larvae, and they are consistent with the hypothesis that the chemical similarity of hosts is an essential parameter of the host range of taxonomic specialists (Gauld, 1988; Vet and Dicke, 1992). One alternative explanation for restricted host ranges is that host range is a function of responses to habitat or food plant (Gauld, 1988). Because *T. pennator* is not known to attack other Lepidoptera that share food plants with the Papilionidae, this hypothesis cannot provide a complete explanation of its host restriction; moreover, it is invalidated by the finding that responses to plant odors are learned. Another possible explanation is that host range may be a function of physiological compatibility (Brodeur and Vet, 1995). This hypothesis is compatible with the two behavioral hypotheses but would be more compelling had no support been found for the others. Possible physiological mechanisms underlying host range have not been studied in *Trogus*. However, given the evidence found in this study for behavioral correlates with host range, it is unlikely (at least among the potential host species studied) that physiology is an important determinant of host use.

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SEED CHEMISTRY OF *Sophora chrysophylla* (MAMANE) IN RELATION TO DIET OF SPECIALIST AVIAN SEED PREDATOR *Loxioides bailleui* (PALILA) IN HAWAII

P. C. BANKO,[1,*] M. L. CIPOLLINI,[2] G. W. BRETON,[3] E. PAULK,[2] M. WINK,[4] and I. IZHAKI[5]

[1]*U.S. Geological Survey, Pacific Island Ecosystems Research Center*
Kilauea Field Station, P.O. Box 44
Hawaii National Park, Hawaii 96718

[2]*Department of Biology*
Berry College
P.O. Box 430, Mount Berry, Georgia 30149

[3]*Department of Chemistry, Berry College*
P.O. Box 5016, Mount Berry, Georgia 30149

[4]*Institut für Pharmazeutische Biologie*
Universitat Heidelberg
Im Neuenheimer Feld 364
D-69120 Heidelberg, Germany

[5]*Department of Biology*
University of Haifa at Oranim
Tivon 36006, Israel

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Abstract—This study describes the chemical ecology of a tritrophic interaction among species endemic to the island of Hawaii, USA: a tree (*Sophora chrysophylla*: mamane), an endangered bird (*Loxioides bailleui*; palila), and moth larvae (*Cydia* spp.). Palila and *Cydia* both specialize on the seed embryos of mamane but avoid eating the seed coats. Palila actively seek out and feed mamane embryos and *Cydia* larvae to their nestlings. Because mamane embryos contain potentially toxic levels of alkaloids, including broadly toxic quinolizidine alkaloids, and because insects often sequester alkaloids from their food plants, we focus on the questions of why palila forage upon mamane embryos and why they supplement their diet with *Cydia* larvae. Our data show that mamane embryos contain high amounts of potentially toxic alkaloids, but are well balanced nutritionally and contain lipids, carbohydrates, proteins, amino acids, and minerals at levels that are likely to be sufficient for maintenance and breeding. Mamane seed coats contain lower levels of alkaloids and nutrients, somewhat

* Author to whom correspondence should be addressed. E-mail: paul_banko@usgs.gov

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higher levels of phenolics, and much higher levels of nondigestible fiber. Taken together, these results suggest that palila have evolved tolerance to high levels of alkaloids and that they forage upon embryos primarily because of their availability in the habitat and high nutritional reward. Our data also suggest that *Cydia* are used by palila because they are readily accessible, nontoxic, and nutritious; the larvae apparently do not sequester alkaloids while feeding upon mamane seeds. Our results are interpreted with respect to the likelihood of current and historical coadaptive responses in this ecologically isolated and simplified island setting.

Key Words—Coadaptation, *Cydia*, Hawaii, *Loxioides bailleui*, mamane, nutrition, palila, quinolizidine alkaloids, seed predation, secondary metabolites, *Sophora chrysophylla*, toxins.

INTRODUCTION

During much of the year, the US federally listed endangered bird palila (*Loxioides bailleui*; Drepanidinae) feeds primarily on developing seeds of mamane [*Sophora chrysophylla* (Salisb.) Seem; Fabaceae]; both species are endemic to the Hawaiian Islands and occur together in the subalpine dry forest of Mauna Kea Volcano on the island of Hawaii, USA. Palila adjust their breeding according to annual variation in the timing and abundance of mamane seeds (van Riper, 1980a; Pratt et al., 1997; Hess et al., 2001; Banko et al., 2002a). Palila feed on mamane by tearing open the pods, extracting immature seeds from their seed coats, and consuming some or all of the embryos. Mamane embryos also are fed to developing young by day 3, but as early as day 2 (van Riper, 1980a), and this diet is supplemented by insect larvae, mamane flower parts, and a few other items (Banko et al., 2002a). The only other species that we have observed eating mamane seeds are larvae of specialist moths, *Cydia* spp. (Lepidoptera: Tortricidae), upon which both adult and nestling palila commonly feed as a supplement to mamane (Banko et al., 2002a). Because whole seeds of mamane and other species of *Sophora* have been reported to contain levels of quinolizidine alkaloids (QAs) that are potentially lethal to vertebrates (Kadooka et al., 1976; Murakoshi et al., 1984), and because insects feeding upon alkaloid-containing plants often sequester toxic levels of plant metabolites, the ability of palila to specialize upon these resources is especially intriguing.

This study addressed some questions related to the specialized feeding behavior, primarily from the bird's perspective, but also from the plant's and insect's perspectives. We had three goals. The first was to describe the chemical makeup of mamane seed coats and embryos as a means of understanding the nutritional and secondary chemical factors that induce palila to consume embryos but discard seed coats. In particular, we wanted to know if embryos at the stage consumed by palila are relatively less toxic or more nutritious than the seed coats. The second goal was to compare the nutritional composition of mamane embryos with the

nutritional requirements of wild birds as a means of understanding why palila might exhibit such dietary restriction. That is, considering nutritional parameters alone, are mamane seeds particularly well balanced in composition, especially with regard to rearing young on such a restricted diet? Our third goal was to determine the chemical composition of *Cydia* larvae as a means of explaining why palila might also exploit this resource. Are *Cydia* larvae simply an accessible, nontoxic source of nutrition for palila, or do the insects sequester mamane toxins within their bodies at levels potentially toxic to birds? If the latter, why are palila able to feed upon these larvae?

If significant levels of QAs or other secondary metabolites are present in mamane embryos, we predicted that embryos must represent a profitable and nutritionally balanced reward. This would be particularly true if there are costs associated with the bird's ability to tolerate high levels of secondary chemicals in the embryo. Second, based upon the lack of aposematic coloration, we predicted that *Cydia* larvae would not sequester alkaloids and, thus, would likely represent to palila a relatively nontoxic and readily exploitable nutritional resource. Cryptic coloration such as that exhibited by *Cydia* is rarely found in insects that sequester toxins from their food plants (cf., Montllor et al., 1990).

We focused on QAs because of their widely reported effects upon organisms ranging from bacteria to insects to vertebrates. QA biosynthesis from lysine via cadaverine to sparteine/lupanine takes place in the aerial green parts of legumes (Wink et al., 1982; Wink, 1987b) followed by translocation via the phloem to other plant parts (Wink and Witte, 1991), where they accumulate predominantly in the vacuoles of epidermal and subepidermal cells (Wink et al., 1984). Especially rich in alkaloids are the seeds, which can contain up to 8% dry mass (Wink, 1993).

It has been shown experimentally that pure QA or QA mixtures are broadly biologically active (for review of the older literature see Kinghorn and Balandrin, 1984) in that they inhibit the multiplication of potato-X virus (Wink, 1987a) and the growth of gram-negative and gram-positive bacteria (Wink, 1984a; Tyski et al., 1988) and certain fungi (Wippich and Wink, 1985). QAs deter feeding of a number of herbivores (nematodes, insects, molluscs, mammals) or are directly toxic or mutagenic to them (Cantot and Papineau, 1983; Bentley et al., 1984; Wink, 1984b; Dreyer et al., 1985; Johnson and Bentley, 1988; Matsuda et al., 1989; Knauer et al., 1995; Zhao et al., 1998; for review see Wink, 1992, 1999). Toxic effects are probably due to inhibition of Na^+ and K^+ ion channels to interactions with the nicotinic and muscarinic acetylcholine receptors, and to inhibition of protein synthesis (Wink and Twardowski, 1992; Wink et al., 1998; Wink, 2000). By targeting several phylogenetically conserved receptor sites, QAs provide a defense against a broad spectrum of herbivores and microorganisms (Wink, 1998a,b).

METHODS AND MATERIALS

Study Site

We collected mamane seeds and *Cydia* larvae in subalpine forest on the western slope of Mauna Kea, Hawaii (Figure 1), where over 90% of the palila population has become concentrated since before 1975 (van Riper et al., 1978; Jacobi et al., 1996; Gray et al., 1999). The forest on the western slope extends

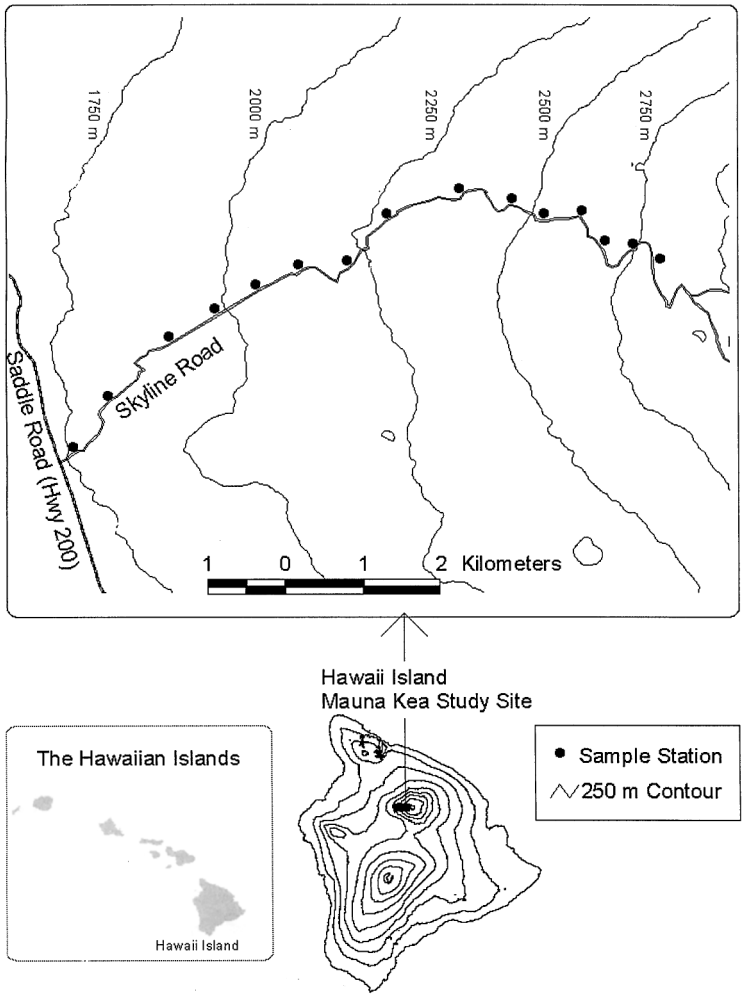


FIG. 1. Map of the study area on the western slope of Mauna Kea, Hawaii.

from about 1750 to 2900 m elevation and is cool and dry, receiving an average of 511 mm of rain annually (Juvik et al., 1993). The study area is dominated by only two tree species: mamane, which occurs in single-species stands over a portion of the western slope, and naio (*Myoporum sandwicense*), which generally dominates mamane in mixed stands (Scott et al., 1986; Hess et al., 1999). Tree heights rarely exceed 8 m. Where mamane and naio occur together, canopy cover may exceed 60%, but overall the forest resembles an open-canopy woodland, due largely to the long history of browsing by introduced feral sheep (*Ovis aries*) and mouflon sheep (*O. musimon*). These and other ungulates, such as cattle (*Bos taurus*) and goats (*Capra hircus*), severely browse mamane trees and seedlings. In recent years, however, populations of browsing ungulates have been reduced, and the understory is filling in with young mamane and naio as well as several other endemic shrubs and small trees (Hess et al., 1999). Endemic and alien grasses and other herbaceous species occur throughout the area, but ground cover tends to be most dense at intermediate and lower elevations of the forest. Wildfire has not occurred within our study area for decades and possibly not for a century or longer, but fire is a natural, if infrequent, feature of this ecosystem.

Seed Collection and Preservation

We collected mamane seeds during August 4–18, 1999, along an elevation gradient extending from 1750 to 2820 m. Palila occur throughout the area where we collected, and August represents a time when, in most years, all age classes of birds, including nestlings and fledglings, are consuming mamane seeds. However, by August the mamane seed crop has largely matured (pods have turned brown) and immature seeds (in green pods) are becoming increasingly scarce (van Riper, 1980b; Banko et al., 2002b). We arbitrarily picked 6–34 full-sized, green pods from 75 trees ($>$2 m tall $\times$ $>$2 m wide) at 15 stations (5 trees/station) separated by 76 m of elevation. Pods ($N = 1236$) contained 1–10 expanded seeds each, but some seeds were partially or entirely eaten by *Cydia* larvae or were destroyed by microorganisms or other agents. We extracted seeds that were expanded ($\geq$3 mm across the broad surface of the pod), removed their seed coats, and flash-froze (liquid nitrogen) the embryos and seed coats in separate vials within an hour of collection. Samples were stored at -70°C until shipped in liquid nitrogen to Berry College, Georgia, USA, for freeze-drying and analysis.

Cydia Collection and Preservation

We collected 200 *Cydia* larvae from mamane pods between 2000 and 2500 m elevation during September 21–November 2, 1995. Specimens were flash-frozen in liquid nitrogen within 5 min of being extracted and were stored at -70°C until shipped in liquid nitrogen to Berry College for freeze-drying and analysis. In

order to determine if alkaloids within whole *Cydia* are mainly associated with gut contents, we collected a second group of 209 larvae during May 13–July 19, 2000 using similar methods, but fasted them for 24 hr prior to flash-freezing.

Sample Preparation and Storage

Immediately upon receipt from Hawaii, embryos, seed coats, and *Cydia* larvae were freeze-dried, ground to pass a #40 mesh screen, and stored in a desiccating chamber at -20°C.

Nutritional Analyses

We analyzed freeze-dried embryo, seed coat, and *Cydia* samples for: (1) total reducing sugars by ethanol extraction followed by the anthrone assay (Smith, 1981), (2) total nonstructural carbohydrate by α-amylase extraction followed by the anthrone assay (Spiro, 1966; Smith, 1981), (3) total lipids by petroleum ether extraction (Williams, 1984), (4) amino acids by hydrolysis followed by ion-exchange chromatography (Moore and Stein, 1951) using a LC5000 Biotronic amino acid analyzer, (5) mineral nutrients by acid hydrolysis followed by inductively-coupled plasma emission spectroscopy, and (6) total nitrogen by acid hydrolysis and direct N detection (Williams, 1984). Total protein was estimated three ways: (1) by using the Bradford assay (Jones et al., 1989), (2) by summing total amino acids, and (3) by multiplying total nonalkaloidal nitrogen by 6.25 (Williams, 1984).

Secondary Chemical Analyses

QA Extraction and Sample Clean-Up. Freeze-dried embryo, seed coat, and *Cydia* samples were extracted by weighing subsamples (500 mg) into 40 ml centrifuge tubes, to which 15 ml 0.1 N HCl was added, followed by a 5-hr room-temperature incubation. The sediment was suspended using an ultrasound bath, followed by centrifugation at 4800 rpm for 10 min. The supernatant was collected, and the pellet was reextracted with another 15 ml 0.1 N HCl. Following a second centrifugation, the supernatant was collected, combined with the first extract, and made alkaline with 6 N NaOH (to pH 13). Total volume of the alkaline extracts was determined by weighing, and aliquots (18 ml) were placed on an Extrelut NT 20 (Merck) column for solid–liquid extraction. The aqueous phase was allowed to adsorb onto the column, then three sequential 20-ml aliquots of Cl_2CH_2 were added and allowed to elute. The combined Cl_2CH_2 eluates were collected and reduced to dryness in a rotary vacuum evaporator. The residue was collected in about 5 ml Cl_2CH_2 and transferred quantitatively into a test tube (10 ml). After the Cl_2CH_2 was removed at room temperature under nitrogen, the alkaloidal residue was taken up with 0.5–7.5 ml of methanol and was stored at -20°C.

QA Qualitative GLC-MS Analysis. A bulk seed embryo extract (containing all alkaloids) was analyzed qualitatively by using a Carlo-Erba 4160 GC coupled to a Finnigan MAT 4500 quadrupole mass spectrometer, and with a Supelco OV-1 microcapillary column (30 m long $\times$ 0.25 mm ID; 0.25-μm-thick coating). The following conditions of analysis were employed: injection volume: 1 μl; split ratio: 1:20; carrier gas: He; flow rate: 1 ml/min; injector temperature: 250°C; temperature program: 120°C, 3 min isothermal, 120–312°C at 10°C/min, 10 min isothermal. The electron impact mass spectra were recorded at 45 eV ionization energy by an INCOS data system. Major GLC peaks were identified by comparison of their MS spectra with published data and with an authenticated library of MS spectra.

QA Quantitative GLC Analysis. Bulk embryo, seed coat, and *Cydia* extracts were analyzed quantitatively for major alkaloids by using an HP5890A gas chromatograph, equipped with a flame ionization detector and an OV-1 column identical to that used for qualitative GLC assays. Conditions of analysis were: injection volume: 4–10 μl; split ratio: 6.5:1; injector temperature: 300°C; carrier gas: He at 10 psi; temperature program: 120°C, 3 min isothermal, 120–312°C at 10°C/min. Cytisine (Sigma) was used as an external standard (0–3.5 mg/ml, slope = 0.2495, $R^2 = 0.990$). Major alkaloid peaks in routine GLC assays were identified by reference to the qualitative GLC-MS chromatographic data.

Total Phenolics. Embryo, seed coat, and *Cydia* samples were analyzed for total phenolics by using ethanol extraction followed by the Prussian blue colorimetric assay (Budini et al., 1980; Graham, 1992).

Statistical Treatment

All quantitative analyses were run in triplicate, and the results are reported as percent of dry mass plus or minus one standard deviation, except for trace minerals, which are reported in parts per million. Because variances associated with these determinations were an estimate of measurement error (individual samples were bulked prior to analysis to achieve sufficient sample size), statistical comparisons among the three tissue types were not appropriate (Sokal and Rolhf, 1981). The data were intended to be interpreted in an exploratory fashion, rather than being used to test any a priori hypotheses.

RESULTS

Identification of Major QAs

Based upon qualitative GLC-MS data, we confirmed the identity of about 15 compounds in the alkaloidal extract of mamane embryos (Table 1; Figure 2), primarily QAs and the bipiperidyl alkaloid ammodendrine. Major constituents

TABLE 1. RESULTS OF QUALITATIVE GLC-MS ANALYSIS OF COMPOUNDS IN MAMANE EMBRYOS

Alkaloid[a]	Retention time (min)	Retention index[b]	Total alkaloids (%)
1. Sparteine	9.1	1790	≤0.1
2. 11,12-Dehydrosparteine	10.1	1840	≤0.1
3. Ammodendrine	10.6	1865	≤0.1
4. Lusitanine	11.2	1898	≤0.1
5. *N*-Methylcytisine	12.4	1955	5.5
6. Cytisine	13.3	1995	41.5
7. 5,6-Dehydrolupanine	15.5	2140	13.0
8. Rhombifoline	16.1	2160	≤0.1
9. Lupanine	16.31	2170	12.8
10. 11-Allylcytisine	17.4	2250	≤0.1
11. Matrine	18.0	2293	4.3
12. *N*-Formylcytisine	18.6	2330	≤0.1
13. Anagyrine	19.6	2380	21.0
14. Phthalate[c]	21.2	2493	≤0.1
15. Baptifoline	23.4	2650	≤0.1

[a] Numbers correspond with labeled peaks in Figure 2.
[b] Determined via co-chromatography with a mixture of linear alkanes of known molecular weights.
[c] Phthalate is a contaminant from the laboratory solvent and is not an alkaloid.

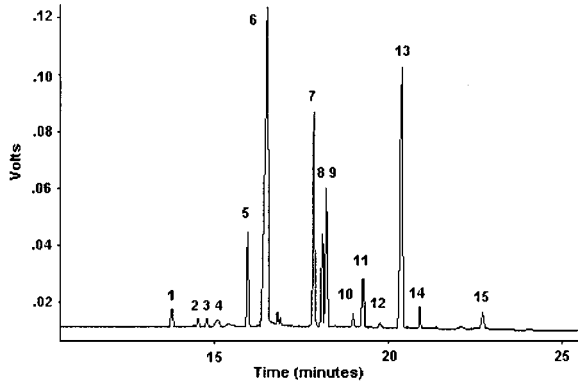


FIG. 2. Sample GLC chromatogram showing the separation of the 15 most important compounds in a sample of mamane seed embryos. Numbered peaks correspond to compounds identified via GLC-MS and listed in Table 1 [note that phthalate (peak 14) is a contaminant from the laboratory solvent and is not an alkaloid]. For conditions of analysis, see the text.

TABLE 2. CARBOHYDRATE, LIPID, AND PROTEIN CONTENT OF MAMANE EMBRYOS, SEED COATS, AND *Cydia* LARVAE

Nutrient	Percent of dry mass ± 1 SD		
	Embryos	Seed coats	*Cydia*
Total reducing sugars	4.92 ± 0.52	6.32 ± 0.43	0.77 ± 0.57
Total other nonstructural carbohydrates	12.42 ± 0.33	15.87 ± 0.71	4.10 ± 5.00
Total lipids	29.69 ± 0.05	0.18 ± 0.10	62.24 ± 2.85
Total protein via Bradford assay	32.81 ± 2.31	11.69 ± 1.24	41.01 ± 2.46
Total protein via total amino acids	27.12 ± 0.59	9.97 ± 0.32	
Total protein via total N	26.29 ± 0.07	9.92 ± 0.21	

included cytisine, 5,6 dehydrolupanine, lupanine, and anagyrine, which together comprised about 88% of the total alkaloids. These data confirm the presence of significant levels of QA alkaloids in mamane embryo tissues fed upon by palila.

Comparison of Mamane Embryos and Seed Coats

Nutrients. In comparison with seed coats, embryos contained slightly less nonstructural carbohydrate and trace minerals, but much higher levels of total lipids, total protein (by all three measures), and most major minerals (Tables 2 and 3). Reflecting their higher level of total protein, mamane embryos were much

TABLE 3. MINERAL CONTENT OF MAMANE EMBRYOS AND SEED COATS

Mineral	Embryos	Seed coats
Major minerals (percent of dry mass ± 1 SD)		
N	4.77 ± 0.01	1.72 ± 0.04
P	0.37 ± 0.00	0.14 ± 0.00
K	0.83 ± 0.01	0.87 ± 0.00
Ca	0.19 ± 0.00	0.32 ± 0.00
Mg	0.22 ± 0.00	0.07 ± 0.00
Total major minerals	6.38 ± 0.01	3.13 ± 0.01
Minor minerals (ppm ± 1 SD)		
Mn	19.67 ± 0.47	61.33 ± 0.47
Fe	57.33 ± 1.25	50.67 ± 0.47
Cu	13.67 ± 0.47	4.33 ± 0.47
B	45.00 ± 0.82	31.33 ± 0.47
Al	9.33 ± 0.47	31.67 ± 0.47
Zn	44.67 ± 0.47	39.33 ± 0.47
Na	20.00 ± 0.00	27.00 ± 0.82
Total minor minerals	209.67 ± 0.56	245.67 ± 0.52

TABLE 4. AMINO ACID CONTENT OF MAMANE EMBRYOS AND SEED COATS

Amino acid	Percent of dry mass ± 1 SD	
	Embryos	Seed coats
Cysteine	0.36 ± 0.06	0.17 ± 0.03
Aspartate	2.99 ± 0.04	1.16 ± 0.01
Methionine	0.28 ± 0.03	0.14 ± 0.01
Threonine	1.01 ± 0.01	0.46 ± 0.01
Serine	1.47 ± 0.02	0.63 ± 0.00
Glutamate	5.49 ± 0.10	1.33 ± 0.02
Proline	1.18 ± 0.09	0.60 ± 0.02
Glycine	1.29 ± 0.04	0.69 ± 0.02
Alanine	1.09 ± 0.05	0.55 ± 0.03
Valine	1.15 ± 0.08	0.51 ± 0.05
Isoleucine	1.24 ± 0.06	0.46 ± 0.04
Leucine	2.06 ± 0.04	0.75 ± 0.03
Tyrosine	1.33 ± 0.04	0.47 ± 0.02
Phenylalanine	1.07 ± 0.06	0.43 ± 0.01
Histidine	0.70 ± 0.00	0.27 ± 0.01
Lysine	1.74 ± 0.04	0.72 ± 0.02
Argininine	2.69 ± 0.09	0.61 ± 0.03

higher than seed coats in all amino acids measured (Table 4). Amino acid profiles for embryos were relatively balanced, ranging from glutamate at 5.5% to methionine at 0.28% dry mass. We detected no nonprotein (toxic) amino acids in our analysis. Embryos were much lower than seed coats in nondigestible fiber (9.47% vs. 57.55%, respectively, based upon subtraction of all measured nutrient and secondary chemical constituents from 100%).

Secondary Metabolites. In comparison with seed coats, mamane embryos contained much higher levels of all major alkaloids as well as several other individual metabolites (Table 5); embryos also contained about four times greater levels of total alkaloids (about 4% of dry mass). The results suggest the opposite for total phenolics; embryos were nearly eight times lower in total phenolics than seed coats, which contained about 4% of dry mass (Table 5).

Comparison of Mamane Embryos and Cydia Larvae

Nutrients. In comparison with mamane embryos, whole *Cydia* larvae contained slightly lower amounts of total sugars, but higher amounts of total lipid and total protein (Table 2). There was essentially no digestible fiber in *Cydia*, in that our estimates of major nutrients exceeded 100% when summed. Due to insufficient sample material, we did not assay *Cydia* for minerals.

TABLE 5. ALKALOID AND TOTAL PHENOLIC CONTENT OF MAMANE EMBRYOS AND SEED COATS AND *Cydia* LARVAE FROZEN IMMEDIATELY FOLLOWING COLLECTION

Alkaloid	Percent of dry mass ± 1 SD		
	Embryos	Seed coats	*Cydia*
Sparteine	0.07 ± 0.01	0.00 ± 0.00	0.001 ± 0.000
Ammodendrine	0.02 ± 0.01	0.01 ± 0.00	0.000 ± 0.000
N-Methylcytisine	0.05 ± 0.01	0.05 ± 0.01	0.002 ± 0.000
Cytisine	1.56 ± 0.1	0.2 ± 0.03	0.002 ± 0.000
5,6-Dehydrolupanine	0.56 ± 0.07	0.11 ± 0.02	0.001 ± 0.000
Lupanine	0.78 ± 0.04	0.23 ± 0.01	0.002 ± 0.000
Matrine	0.18 ± 0.04	0.07 ± 0.01	0.001 ± 0.000
Anagyrine	1.01 ± 0.16	0.4 ± 0.01	0.001 ± 0.000
Baptifoline	0.06 ± 0.01	0.01 ± 0.00	0.000 ± 0.000
Total alkaloids	4.29 ± 0.05	1.08 ± 0.01	0.087 ± 0.010
Total phenolics	0.57 ± 0.13	4.31 ± 0.10	2.84 ± 0.33

Secondary Metabolites. In comparison with mamane embryos, the sample of freshly collected *Cydia* larvae contained much higher amounts of total phenolics (about 5 times higher; Table 5). These levels (about 3%) were similar to the levels found in mamane seed coats. These larvae contained much lower levels of alkaloids, however. Total alkaloid content was 0.087% of dry mass, which was about 50 times lower than mamane embryos. Sparteine, *N*-methylcytisine, cytisine, 5,6-dehydrolupanine, rhombifoline, lupanine, matrine, and anagyrine were found at levels of 0.001–0.002% of dry mass (Table 5), along with numerous apparent breakdown products at similarly low levels (data not shown). Residual alkaloids and breakdown products were largely eliminated with fecal matter, as total alkaloid levels were reduced to a level (0.019%) about 8 times lower during the 24-hr fasting period. The only identifiable alkaloid in the sample of starved *Cydia* was sparteine at 0.002% of dry mass. Altogether, alkaloid levels in fasted larvae were about 200 times lower than levels in seed embryos, suggesting that these insects do not sequester alkaloids in their tissues while feeding upon mamane.

DISCUSSION

General Observations. Why do palila feed commonly on mamane embryos while consistently excluding seed coats? Embryos at the stage consumed by palila contained high levels of several different alkaloids, including the highly toxic QA cytisine, which exceeded 1.5% of seed dry mass. They contained about 4% total alkaloids, whereas seed coats had lower levels of the same major alkaloids (about 1% total alkaloids). The major compound (cytisine) and the overall concentration

TABLE 6. TOTAL DAILY ALKALOID INTAKE BY PALILA FORAGING ON MAMANE SEEDS COMPARED WITH LITERATURE REPORTS FOR TOXICITY IN ANIMALS

Parameter			Value
Palila body mass (kg)[a]			0.0377
Seeds consumed per day (N)[b]			300
Dry seed mass consumed per day (g)			10.67
Total alkaloids consumed per day (mg)			457.7
Oral dose alkaloids per day (mg/kg body mass)			12141
Quinolizidine alkaloid LD_{50} oral toxicity in animals (mg/kg)[c]			
Compound		Species	LD_{50}
Cytisine		Mouse/rat	50–500
Sparteine		Mouse	350–510
N-Methylcytisine		Rat	>2500

[a] Jeffrey et al. (1993).
[b] US Geological Survey, unpublished data.
[c] Barlow and McLeod (1969), Hatfield et al. (1977), Wink (1998b).

of alkaloids in mamane embryos are comparable to *Sophora secundiflora* Lag. ex DC (mescalbean), a species with well-known broad toxicity (Keller, 1975; Keller et al., 1976). While the toxicity of *S. secundiflora* has been attributed to synergism among alkaloids and toxic amino acids (Izaddoost et al., 1976; Hatfield et al., 1977), we found no toxic amino acids in mamane. Because embryos contained levels of alkaloids known to cause severe neurological symptoms and death in humans and other animals, it is likely that palila have evolved a tolerance to the alkaloids. Based upon an estimated daily consumption rate of 300 seeds per adult palila (US Geological Survey, unpublished data), we calculated a total daily alkaloid intake of about 12,000 mg/kg body mass (Table 6). This value far exceeds oral LD_{50} estimates for small granivorous animals such as mice. The tolerance of palila to such high levels of alkaloid may be the product of intestinal uptake followed by detoxification in the liver (with consequent effects on relative liver size), nonabsorption from the gut (unlikely because QAs are lipophilic under alkaline conditions of the gut and should diffuse into the body), or active export via P-glycoprotein-type ABC transporters.

Relative to embryos, seed coats had higher levels of phenolics, but lower levels of most major nutrients and higher levels of fiber. Thus, it is likely that seed coats are rejected because of lower overall nutritional value, coupled perhaps with a negative influence of phenolic-based secondary metabolites. Phenolic compounds, such as tannins and phenolic glycosides that may be found in the seed coats of legumes, can have both direct deterrent effects through toxicity or astringency and indirect effects upon digestion via their ability to bind to complex molecules such as proteins (cf., Levey and Cipollini, 1999). To avoid seed coats, palila must

spend time and effort extracting the embryos. After the pod has been torn open, the exposed portion of the seed coat is removed before the embryo is extracted; the remainder of the seed coat is left in the pod, which is discarded after the embryo has been consumed. However, palila in the hand are capable of removing the seed coat in an entirely different manner. When presented with an intact seed that has been removed from the pod, a hand-held palila takes the seed in its bill, orients the long axis of the seed with the long axis of its bill, and bites through the seed coat (P. Banko, personal observation). The bird then rotates the seed inside its bill with its tongue, causing the seed coat to neatly peel away in one piece and fall from the side of the bird's bill. The exclusion of the seed coat appears to be purposeful and not an accident of sloppy eating.

Are mamane embryos a particularly well-balanced food for palila? Based upon our understanding of avian nutrition (cf., Robbins, 1993), mamane embryos seem well balanced nutritionally and contain relatively low levels of fiber and high levels of carbohydrate, lipid, and protein (a ratio of about 1:2:2), as well as relatively balanced profiles of essential amino acids and minerals. For example, based on our estimate of daily seed consumption, the amount of nitrogen and protein consumed is, respectively, about 14 and 9 times that required for maintenance in granivorous birds (Table 7). While variation in digestive efficiency is not considered in these estimates, and we do not have an estimate of nitrogen and protein requirements for palila, it is unlikely that palila have difficulty obtaining sufficient protein or other nutrients from mamane seeds. Although we did not assay minor

TABLE 7. DAILY PROTEIN AND N INTAKE BY PALILA FORAGING ON MAMANE SEEDS, WITH RESPECT TO PROTEIN AND N REQUIREMENTS FOR MAINTENANCE IN GRANIVOROUS BIRDS

Parameter	Value
Dry mass per embryo (g)	0.0356
N in dry embryo (%)	4.77
Protein in dry embryo (%)	32.81
Total N per embryo (g)	0.0017
Total protein per embryo (g)	0.0117
Total N in 300 embryos (g)	0.5098
Total protein in 300 embryos (g)	3.5041
Palila body mass (kg)[a]	0.0377
Required N intake per day per bird (g)[b]	0.0364
Required protein intake per day per bird (g)[b]	0.3961
Ratio of consumed/required N	13.9973
Ratio of consumed/required protein	8.8465

[a] Jeffrey et al. (1993).
[b] Averages of estimates for granivorous birds similar in body mass to palila (Weglarczyk, 1981; Murphy, 1993; Robbins, 1993).

organic constituents, such as vitamins, mamane embryos are particularly well balanced nutritionally, thus largely explaining why palila rely so heavily on mamane embryos for their daily diet and for rearing their offspring. We hypothesize that palila evolved specialization on mamane as a result of food competition with other seed-eating honeycreepers (Drepanidinae) in relatively simple habitats that were dominated by only a few tree species.

Why do palila commonly supplement their diets with *Cydia* larvae? In addition to being found conveniently inside mamane pods, the data suggest that these insects are relatively nontoxic sources of lipid, protein, and other nutrients and, thus, represent occasional but relatively high-quality resources for the bird. Adults likely seek out *Cydia* to feed to their chicks, as the incidence of *Cydia* mandibles is high in nestling and fledgling fecal samples (US Geological Survey, unpublished data). Relatively nontoxic nutritious foods would be important to the survival and development of birds that were susceptible to alkaloid toxicity. The frequency of *Cydia* mandibles is also higher in fecal samples collected from adults than from juvenile birds that are independent of parental care, suggesting that experience may be helpful in finding pods containing larvae. For example, birds may learn to identify pods that contain *Cydia* by the presence of silk caps that are spun by the larvae over their emergence holes, and they may learn that some trees support larger infestations of larvae than do others. Although *Cydia* larvae have a much higher level of phenolics than do seed embryos, total phenolics in the insects were close to the level found in seed coats. Thus, our conclusion that phenolics might induce palila to reject seed coats must be tempered by an understanding of the relative nutritional rewards. Assuming that the phenolics within *Cydia* bodies are of mamane origin and have similar antinutritional effects when consumed by palila regardless of the food source, seed coats should be nonetheless less nutritious than are *Cydia* larvae.

The data suggest that *Cydia* larvae break down the alkaloids in the course of digestion, as evidence of alkaloids and breakdown products is found primarily in insects containing material within the gut. We found no evidence that *Cydia* larvae sequester significant quantities of these alkaloids in their bodies, thus supporting our prediction that crypsis should be associated with a lack of toxin sequestration. Furthermore, crypsis may reduce predation by palila since pods often contain larvae that have consumed one or more embryos and are, thus, relatively exposed to foraging birds. *Uresephita polygonalis virescens* (Lepidoptera: Crambidae), an indigenous or possibly endemic moth whose aposematically colored larvae feed upon mamane foliage, is more likely to sequester alkaloids, based upon studies of a congener, *U. reversalis*, which feeds upon a QA-containing legume (Montllor et al., 1990). Historically, *U. polygonalis* larvae were major prey of adult palila apparently on Mauna Loa (Perkins, 1903, 1913), where the birds are now extinct (Scott et al., 1986). *U. polygonalis* is presently rare on Mauna Kea and is probably seldom eaten by palila, even though the birds forage generally for insects and

spiders on mamane foliage and branches in addition to extracting *Cydia* from pods (US Geological Survey, unpublished data).

Questions Resulting from This Analysis. Are nonspecialist birds and other generalist seed predators more susceptible to mamane seed alkaloids than are palila and *Cydia*? In other words, is there any evidence for evolved tolerance in these specialist species? Because the palila is a US federally listed endangered species, which limits work with captive birds, it be may difficult to compare its tolerance to QA alkaloids with that of related bird species in side-by-side feeding trials. Nevertheless, controlled feeding trials with other species might indirectly address part of the question. We have been unable to find published reports on oral toxic effects of QAs on wild birds; however, four 10- to 14-day-old, hand-reared house finches (*Carpodacus mexicanus*) convulsed and died within about 10 min of ingesting mashed mamane seeds (C. Kuehler, Keauhou Bird Conservation Center, personal communication). Assuming that a suitable laboratory diet can be developed, feeding trials with *Cydia* and related moth species are a promising possibility.

Palila and *Cydia* forage heavily upon certain trees and lightly upon others (US Geological Survey, unpublished data); therefore, work is needed to determine what factors influence selection among individual mamane plants. Are seeds of preferred individuals particularly high in certain required nutrients (lipids, carbohydrates, protein, essential amino acids, etc.), or are they particularly low in certain toxic secondary metabolites? Do *Cydia* compete with palila for mamane seeds, and what are the consequences of this interaction? Answers to these questions may help explain the evolutionary impact on mamane of seed predation by palila and *Cydia*. It is possible that individual mamane trees vary in their seed alkaloid profiles due to both genetic and environmental effects. For example, samples of seeds collected from two different individual plants in Hawaii have been reported to contain virtually no cytisine, instead being dominated by anagyrine, *N*-methylcytisine, and baptifoline (Pena and Cassels, 1996), whereas our bulk sample derived from many individuals was dominated by cytisine. Considering possible inter-individual variation, future research might focus on relating levels of alkaloids and other chemical constituents of seeds with foraging preferences of palila and rates of *Cydia* seed predation within individual mamane trees. If secondary chemistry substantially influences palila foraging and moth oviposition patterns, we would expect strong selection pressure for seed embryo toxicity in mamane. Such a result may indicate that palila are able to reduce their exposure to toxins by adjusting their foraging behavior. In any case, our general conclusion is that continued study of the palila–mamane–*Cydia* triad holds promise for heightening our understanding of plant–animal coadaptation and for understanding the management implications of such interactions. These interacting species can be studied in a relatively simple and isolated environment, where one can better account for factors that might confound or obscure patterns in more diverse environments.

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A LECTIN-LIKE WHEAT GENE RESPONDS SYSTEMICALLY TO ATTEMPTED FEEDING BY AVIRULENT FIRST-INSTAR HESSIAN FLY LARVAE[1]

CHRISTIE E. WILLIAMS,[2,3,*] CHAD C. COLLIER,[2,3]
JILL A. NEMACHECK,[3] CHENGZHI LIANG,[3] and SUE E. CAMBRON[2,3]

[2]*USDA-ARS Crop Production and Pest Control Research Unit*
West Lafayette, Indiana 47907

[3]*Department of Entomology, 100 Smith Hall*
Purdue University
West Lafayette, Indiana 47907

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Abstract—Through gene-for-gene interactions, wheat plants respond to specific biotypes of Hessian fly upon the initiation of first-instar larval feeding. Leaves of plants containing the *H9* resistance gene responded to avirulent biotype L larvae with rapid changes in the levels of several mRNA transcripts and initiation of an incompatible interaction. A low-copy gene, *Hfr-1* (Hessian fly-response gene 1), responded with increased mRNA levels for two days before returning to preinfestation levels by day five. *Hfr-1* mRNA was constitutively expressed in uninfested control plants as well as in plants infested with virulent larvae. The cDNA sequence was similar to a maize gene encoding a β-glucosidase aggregating factor (BGAF), to jacalin-like mannose-binding lectins, and to several plant genes that respond to microbial infections. The potential roles of *Hfr-1* in defending wheat against Hessian fly damage are discussed.

Key Words—Wheat, *Triticum aestivum* L., Hessian fly, β-glucosidase aggregating factor, BGAF, jacalin-like lectin, defense response, resistance, PR gene, incompatible interaction, Genbank number AF483596.

INTRODUCTION

Genetic interactions between the Hessian fly [*Mayetiola destructor* (Say)] and wheat (*Triticum aestivum* L. em Thell.) operate on a gene-for-gene basis (Hatchett and Gallun, 1970; Gallun, 1978), much like the well-documented, race-specific

* To whom correspondence should be addressed. E-mail: christie_williams@entm.purdue.edu
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interactions between microbial pathogens and plants (Bent, 1996). Over 29 resistance genes that protect wheat against 16 biotypes of the Hessian fly have been characterized in wheat and related species (Delibes et al., 1997; Ratcliffe and Hatchett, 1997). The wheat–Hessian fly interaction is very specific; although a wheat genotype may harbor one or more of these resistance genes, the plant becomes resistant only when its resistance gene is able to mediate recognition of the specific biotype of Hessian fly larvae that has infested. Thus, a wheat genotype can be resistant, participating in an incompatible interaction with a biotype of larvae that is avirulent on that wheat genotype. However, the same wheat genotype can become susceptible by participating in an incompatible interaction with a different larval biotype that is virulent on that wheat genotype. The resistant/susceptible phenotype of the plant and the avirulent/virulent phenotype of the insect are interdependent. Plants undergoing incompatible interactions with Hessian fly larvae show little sign of stress or yield loss. However, compatible interactions result in drastic changes in the physiology and development of the wheat plant.

Compatible wheat/Hessian fly interactions (larvae virulent and wheat susceptible) are characterized by the ability of first-instar larvae to feed, grow, and mature between the leaf sheaths near the crown of seedlings, or just above the nodes of older jointed wheat plants. Systemic symptoms in susceptible seedlings include a generalized increase in leaf sheath cell permeability within five days of larval feeding initiation (Shukle et al., 1992), followed by stunting of the plant and chloroplast accumulation, which become evident about five days later (Cartwright et al., 1959; Robinson et al., 1960). A single virulent larva is sufficient to produce these plant symptoms, which become irreversible after four days of larval feeding (Byers and Gallun, 1972). These symptoms are thought to result from injected larval secretions that fail to trigger resistance responses, rather than from consumption of plant material. After five days of larval feeding, the first molt occurs and the second instars are no longer mobile or able to inject secretions into the plant. By this time, changes in the physiology of the plant have provided an environment where nutrients are delivered to the larvae and they are protected by the stunted leaf sheaths.

During incompatible interactions (larvae avirulent and wheat resistant), first-instar Hessian fly larvae fail to increase in length (C. Williams, unpublished data) and die within three to five days of hatching (Painter, 1930). Despite repeated attempts to feed [sites evident in electron micrographs (Grover, 1995)], avirulent larvae fail to establish a permanent feeding site, continue to migrate downward as the plant leaves elongate, and are unable to effect plant stunting and other physiological changes that make the wheat plant a hospitable host for the virulent larvae. In some incompatible interactions, a hypersensitive response develops in the plant (Shukle et al., 1992), but it is unclear whether recognition results in production of insecticidal compounds. However, the plant's ability to detect avirulent larvae does not result in systemic resistance to future infestation. Subsequent feeding by

virulent larvae (tested up to three days after first attempted feeding by the avirulent larvae) leads to obviation of resistance, allowing survival of both virulent and the avirulent larvae that first infested the plant (Grover et al., 1989; J. Stuart, personal communication). The ability of both virulent and avirulent larvae to survive during mixed infestations suggests that avirulent larvae do not induce systemic toxin production. Thus, resistance mechanisms may be highly localized at the attempted feeding sites of avirulent larvae and may not prevent nearby virulent larvae from initiating systemic changes in the plant that result in obviation of resistance.

The complex interactions between the wheat plant and first-instar Hessian fly larvae offer opportunities for molecular analysis of signaling pathways and gene regulation. Biochemical analyses reveal that during a compatible interaction, feeding by virulent larvae results in increased concentrations of carbohydrates in the leaf and crown (Refai et al., 1955; Wellso et al., 1989, 1990) and increased concentrations of Rubisco in the leaf sheath and crown (Shukle et al., 1992). These affected plant tissues are at or near the larval feeding sites. In contrast, little or no visible change is apparent in plants participating in an incompatible interaction after infestation by avirulent larvae, and the biochemical responses are not well defined. The only available molecular information about the resistant plant response documents an increase in 5- and 11-kDa proteins of unknown function that were detected one to two days after attempted feeding by avirulent first-instar larvae. Both proteins were no longer detected by day 5 (Shukle et al., 1992).

Feeding by chewing insects, such as *Manduca sexta* and *Spodoptera littoralis* larvae, results in expression of plant wound-response genes [proteinase inhibitors, polyphenol oxidases (Green and Ryan, 1982; Stout et al., 1996) and leucine aminopeptidase (Pautot et al., 1993)] in addition to a novel set of plant genes (Korth and Dixon, 1997). Although limited information is available about plant responses to insects with piercing mouth-parts, such as the Hessian fly larvae, a few studies have focused on responses to phloem-feeding insects. These insects inflict minimal physical damage to the plant and consequently do not induce wound responses. However, interactions between *Arabidopsis* and the green peach aphid result in dramatically increased expression of pathogenesis-related (PR) genes (Moran and Thompson, 2001) known to be associated with salicylic acid (SA) -dependent responses to microbial pathogens. Whiteflies feeding on tomatoes induce SA-independent, jasmonic acid (JA)/ethylene-dependent genes (Walling, 2000). In addition, silverleaf whiteflies feeding on squash plants induce genes that previously had not been linked to plant–insect interactions: a M20B metallopeptidase-like gene and a β-glucosidase-like gene (van de Ven et al., 2000).

Although the gene-for-gene interactions between wheat and the Hessian fly are genetically well defined at the level of plant phenotypes, lack of information about the defense response at the molecular level led us to search for genes that respond to Hessian fly infestation. We identified *Hfr-1*, a gene whose mRNA levels increased rapidly during an incompatible interaction within 22 hr of egg hatching

and returned to normal within five days. The expression profile of *Hfr-1* corresponded with the critical period in which the first-instar larvae are attempting to establish feeding sites by injecting substances into the plant. Structural similarities between *Hfr-1* and a maize gene encoding a β-glucosidase aggregating factor (BGAF), mannose-binding lectins, and a wheat gene that responds to a chemical elicitor of systemic acquired resistance (SAR), are suggestive of involvement in plant defense against insect attack.

METHODS AND MATERIALS

Insects, Plant Tissues and Infestations. Hessian fly biotype L flies (virulent to wheat resistance genes *H3*, *H5*, *H6*, and the *H7H8* combination, but avirulent to the *H9*-containing variety of wheat used in these experiments) and *vH9* flies (virulent to the *H9*-containing variety of wheat) were maintained in a 4°C cold room as purified laboratory stocks by the USDA-ARS Crop Production and Pest Control Research Unit, Purdue University. According to the methods outlined by Cartwright and LaHue (1944), the fly stocks are increased each year and selected for virulence on appropriate wheat genotypes: biotype L is selected on *H5* wheat, which is resistant to all biotypes maintained in the laboratory except for biotype L, and *vH9* is selected on *H9* wheat. Although wild populations of Hessian fly are composed of mixtures of biotypes, genetically purified insect stocks were used for these experiments so that clearly defined compatible and incompatible interactions could be studied. All wheat plants used in these experiments were of identical genotype, containing the *H9* gene for Hessian fly resistance [variety Iris (Patterson et al., 1994)]. Wheat seedlings were grown in flats of soil in a growth chamber at 20°C and 12-hr photoperiod until they reached the two-leaf stage (7–10 days after germination). During infestations, about 100 newly emerged adult female Hessian flies of biotype L (avirulent on wheat containing *H9*) or *vH9* flies (virulent on *H9* wheat) were allowed to mate and oviposit for 24 hr on flats containing 300 seedlings (Maas et al., 1989). Leaves of each plant were inspected after infestation to ensure the presence of eggs. Plants were grown in chambers at 20°C throughout tissue collection. No other insects or pathogens were apparent on the experimental plants.

RNA Isolation for Differential Display. One week after infestation, leaves were collected above the larval feeding sites, and total RNA was isolated with the kit CLONsep 2 (Clonetech Laboratories, Inc., Palo Alto, California, USA). Differential display experiments were conducted according to the directions in the RNAimage kit 2 (Genhunter Corporation, Nashville, Tennessee, USA). The two *H9* Iris wheat samples used in differential display were of identical age and genotype, but one was infested with *vH9* flies and the other was infested with biotype L flies. Thirty-six unique fragments were identified from reactions of eight primer pairs. Excised cDNA fragments were purified with the Wizard PCR Prep

DNA Purification System (Promega Corporation, Madison, Wisconsin, USA) and were cloned with the pGEM-T System I (Promega). All DNA sequencing utilized infrared fluorescent dye-labeled SP6 and T7 primers on a LiCor Long Reader 4200.

Isolation of a Near Full-Length cDNA. To generate the near full-length *Hfr-1* cDNA clone (UPW1Hfr1a), the partial cDNA sequence, from the 583-bp clone UPW1Hfr1b obtained by differential display of the 3′ end of the *Hfr-1* mRNA, was used as a template for the design of primers. These primers were amplified toward the 5′ end of the mRNA, following the directions of the Smart RACE (Rapid Amplification of cDNA Ends) cDNA amplification kit (Clontech). A total of 1283 bp of sequence was obtained for *Hfr-1* cDNA (Genbank number AF483596). From this sequence, the forward primer (5′-ACGCACACACACAATCCT-3′) and the reverse primer (5′-CAACACCCAGGAACGTACTA-3′) were designed to amplify a 1090-bp fragment from within the 1283-bp of sequence. This amplicon included all of the *Hfr-1* open reading frame. Amplified fragments were isolated from gels by using the Wizard PCR prep DNA purification System (Promega) and were ligated into the pGEM-T Vector (Promega). Plasmid DNA was isolated by using the Wizard SV Plus Miniprep Kit (Promega). The 1283 bp of sequence and the 1090-bp near full-length cDNA clone (UPW1Hfr1a) were used in BLAST inquiries. The 583-bp partial cDNA clone (UPW1Hfr1b) was used to generate a probe for radioactive blot experiments.

Blot Hybridization. For Northern analysis, leaves were collected over a period of eight days, starting three days after infestation (two days before egg hatch), and total RNA was isolated with the Totally RNA kit (Ambion, Inc., Austin, Texas, USA). RNA samples (10–15 μg) were separated on 1.2% formaldehyde–0.8% Agarose denaturing gels in 10$\times$ MOPS buffer (Maniatis et al., 1982). RNA was transferred to Hybond N^+ membranes (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA), hybridized with ^{32}P-labeled *Hfr-1* and 28s rDNA probes. The *Hfr-1* probe was the 583-bp partial cDNA clone (UPW1Hfr1b) encompassing nucleotides 770 through 1283 of the *Hfr-1* sequence. A 650-bp fragment of the barley 28s ribosomal DNA gene was used to detect wheat rRNA in quantification experiments. This clone was kindly provided by J. Anderson (USDA-ARS, West Lafayette, Indiana, USA). The RNA blots were processed according to the Hybond directions. Blots were washed twice with 2$\times$ SSC, 0.1% SDS at 65°C for 30 min. Blots were exposed to Biomax MR film (Eastman Kodak Company, Rochester, New York, USA) with Biotech L-Plus intensifying screens (Fisher Scientific, Pittsburg, Pennsylvania, USA) for three to seven days.

For Southern analysis, genomic DNA was isolated (Osborn et al., 1987) from seedlings of Iris wheat. DNA samples (15 μg) were digested with various restriction enzymes and separated on 0.8% agarose gels in 1$\times$ TBE (Maniatis et al., 1982), blotted onto Hybond N^+ membranes (Amersham Pharmacia Biotech) and processed according to the manufacturer's instructions with a ^{32}P-labeled *Hfr-1* partial cDNA clone.

Sequence Analysis. Database searches were conducted at the NCBI BLAST site (http://www.ncbi.nlm.nih.gov/BLAST/) with the BLASTx option to compare DNA sequences and with the tBLASTx option for deduced translated protein sequences derived from the experimentally determined *Hfr-1* cDNA sequence. Amino acid sequence alignments were performed with Vector NTI Suite (Informax, Inc., Bethesda, Maryland, USA). The program SignalP V1.1 was used to inspect the deduced *Hfr-1* sequence for a potential signal peptide sequence (http://www.cbs.dtu.dk/services/SignalP/#submission). Protein domain analysis was conducted with ProDom (http://prodes.toulouse.inra.fr/prodom/doc/prodom.html).

RESULTS

Response to Infestation. The studies presented here investigated changes in *H9* Iris wheat gene expression during the first 136 hr of attempted feeding by first-instar Hessian fly larvae. All plants included in the study were of identical genotype: homozygous for *H9*, a gene that confers resistance to biotype L but not to the *vH9* strain of Hessian fly. Both compatible and incompatible interactions were studied in *H9* Iris wheat tissue, thus eliminating any variability imposed by use of multiple wheat genotypes.

Seedlings were grown to the two-leaf stage and then divided into three treatment groups. The first group of plants was not infested. Observations of the second group, which was infested with adult *vH9* Hessian flies, confirmed that symptoms of a classical compatible interaction, leading to larval growth and plant stunting, were present by 14 days after infestation. The third group, which was infested with adult biotype L Hessian flies, produced an incompatible interaction leading to larval death and normal plant growth that was indistinguishable from the uninfested controls. Eggs were oviposited on the top surface of the first and second leaf, and both genotypes of larvae hatched simultaneously over a period of a few hours during the morning of the fifth day after infestation. Plant dissections indicated that within a few hours of the first hatch, first-instar larvae had crawled to the base of the culm within the lower leaf sheaths just above the roots. Larvae attempted to feed on the leaf sheath of the second leaf (counting from the bottom toward the top of the plant) and on all tissues that were fully enclosed by sheaths of the first two leaves, including the leaf blade and sheath of immature leaves as well as meristematic tissues. Although Hessian fly larvae begin feeding within a few hours of hatching, they are not synchronous in their establishment of feeding sites at the crown of the plant. Moreover, larval feeding cannot be observed without destroying the plant since it occurs between the leaf sheaths. Thus, the first posthatch time point was taken 22 hr after the prehatch time point, with the exact time of first feeding being unknown. Leaf blades, rather than regions surrounding larval feeding sites, were collected at various times so that systemic gene expression could be monitored.

Expression of Hfr-1. To identify wheat genes responding to attempted-feeding by first-instar Hessian fly larvae, partial cDNA clones were isolated by differential display (Liang and Pardee, 1992). In a comparison of sequences that were differentially expressed between leaves of *H9* Iris wheat plants undergoing compatible versus incompatible interactions, eight primer–pair combinations yielded 36 differentially displayed bands. Several bands representing sequences shorter than 400 bp were not cloned, and 18 sequences returned no BLAST similarities.

A 583-bp cDNA fragment, corresponding to the 3′ end of a differentially expressed wheat gene, referred to here as *Hfr-1* (Hessian fly-response gene 1), was cloned and used as a probe in expression experiments. Northern blot analysis confirmed increased levels of *Hfr-1* mRNA in response to the initiation of feeding by avirulent first-instar larvae on *H9* Iris wheat plants (incompatible interaction; Figure 1). In contrast, the *Hfr-1* gene was expressed at a low constitutive level in leaves of *H9* Iris wheat plants that were either infested with *H9* virulent larvae or were uninfested (compatible interaction and "U"; Figure 1).

After the initiation of avirulent larval feeding, the level of *Hfr-1* mRNA increased in leaf blades for approximately 48 hr and then began to decrease. These avirulent first-instar Hessian fly larvae residing at the base of resistant plants did not increase in length, and they began to die within three days of initiation of feeding, corresponding well with the expression profile of *Hfr-1*. In contrast, only a slight

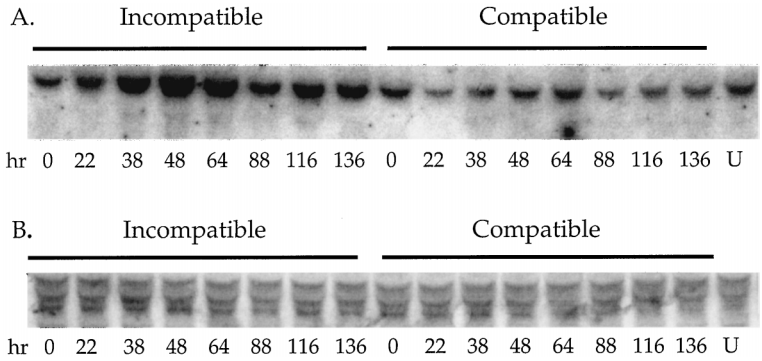


FIG. 1. Accumulation of *Hfr-1* mRNA in response to feeding by Hessian fly larvae. Northern blot analysis was performed on total RNA from leaves of the wheat variety *H9* Iris after infestation by avirulent biotype L (incompatible interaction) and *H9* virulent fly (compatible interaction). "U" indicates mRNA from uninfested control plants. Samples in lanes marked "0" were taken before eggs hatched, but no more than 22 hr before the initiation of larval feeding (time 0). Subsequent samples were taken at the indicated number of hours after the time 0 sample. (A) The blot was hybridized with a ^{32}P-labeled partial cDNA clone of *Hfr-1* (UPW1Hfr1b). (B) The blot was stripped of the *Hfr-1* probe and then rehybridized with a ^{32}P-labeled barley 28s rDNA clone to indicate relative levels of RNA in each lane.

elevation in the level of *Hfr-1* mRNA was observed, during the first 48 hr, in *H9* Iris wheat plants infested with virulent larvae (compatible interaction; Figure 1). Even 10 days after infestation, *Hfr-1* mRNA levels remained low and similar to uninfested controls during this compatible interaction (data not shown). Since the variety Iris is hexaploid (containing three distinct wheat genomes), the mRNA detected by Northern blot analysis may have been contributed by orthologous genes from any of the genomes rather than exclusively by the gene corresponding to the clone UPW1Hfr1b.

Genomic Copies of Hfr-1. Southern blot analysis revealed that *Hfr-1* is a low-copy sequence. DNA from the hexaploid wheat cultivar, Iris, was digested with enzymes that had no restriction site within the *Hfr-1* partial cDNA clone UPW1Hfr1b. The low number of resulting bands (Figure 2) was consistent with only one or a few copies of the *Hfr-1* gene in each of the three wheat genomes. Surprising results from nulli-tetrasomic analysis revealed that *Hfr-1* sequences

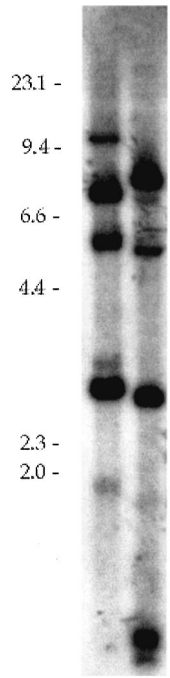


FIG. 2. Genomic copies of *Hfr-1* in hexaploid wheat. *H9* Iris genomic DNA (15 μg/lane) was digested with EcoR I or Dra I restriction enzymes. These enzymes did not cut within the sequence of the *Hfr-1* partial cDNA clone (UPW1Hfr1b). DNA was separated on a 0.8% agarose gel, blotted, and hybridized with the ^{32}P-labeled partial cDNA clone of *Hfr-1*.

were present in only two of the three genomes of the hexaploid line Chinese Spring, with one copy on chromosome 4A and the other on chromosome 7D (Devos and Williams, unpublished results). The banding pattern for restriction digests of Chinese Spring wheat was identical to what was seen for Iris wheat, suggesting that only two loci containing *Hfr-1* sequences were present in the line that was used here for expression studies.

Characterization of Hfr-1 cDNA Sequence and Deduced Amino Acid Sequence. A near full-length cDNA sequence of *Hfr-1*, generated by 5′-RACE, was characterized by DNA sequence analysis. The sequence was 1283 bp long (Genbank number AF483596), including a 5′-untranslated region of 54 bp, a 3′-untranslated region of 194 bp, and a 1035-bp open reading frame encoding a 37.7-kDa protein of 345 amino acids (Figure 3). The predicted protein lacked signal peptide sequences and transmembrane domains.

Database searches and sequence comparisons revealed that the deduced amino acid sequences for the *Hfr-1* gene and the wheat chemically induced gene, *Wci-1* (Genbank number AAC49284.1), were 68% identical (Figure 4a). The chemical benzothiadiazole (BTH) is an analog of salicylic acid. In addition to causing increases in mRNA levels of *Wci-1* and other wheat genes, BTH is an inducer of systemic resistance to powdery mildew, leaf rust, and *Septoria* leaf spot in wheat (Gorlach et al., 1996). The Hfr-1 deduced amino acid sequence was 42% identical to the deduced amino acid sequence encoded by a barley mRNA that is induced by jasmonic acid (Genbank number AAA87042.1) (Lee et al., 1996), a signaling molecule involved in plant abiotic stress and pathogen attack (Farmer and Ryan, 1992; Sembdner and Parthier, 1993). A maize β-glucosidase aggregating factor (BGAF; Genbank number AAF71261.2), which may play a role in defense against insects (Blanchard et al., 2001), was 44% identical to the Hfr-1 deduced amino acid sequence. The similarity between the Hfr-1 deduced amino acid sequence and proteins from rice (Genbank number BAB19404.1), crocus (Genbank number AAG10403.1), hedge bindweed (Genbank number AAC49564), Jerusalem artichoke (Genbank number AAD11577.1), and others that have demonstrated mannose-binding jacalin-related lectin activity (Bourne et al., 1999), strongly suggests a functional role in defense against insects.

The Hfr-1 deduced amino acid sequence appeared to have two domains (Figure 4b). BLAST searches and ProDom analysis indicated that within the first 200 amino acids of the N-terminus lies a domain found in several disease resistance-response proteins of unknown function (ProDom family number 011170; Figure 4b). The sequence from amino acid 201 through 345, at the C-terminus, resembled lectins and myrosinase-binding proteins (ProDom family numbers 000864 and 336299). However, the N-terminal domain that is present in the wheat, maize, and barley proteins is not found in the proteins known to function as lectins (Blanchard et al., 2001). Thus, the Hfr-1 sequence contained a disease resistance-response domain fused to the N-terminus of an entire lectin

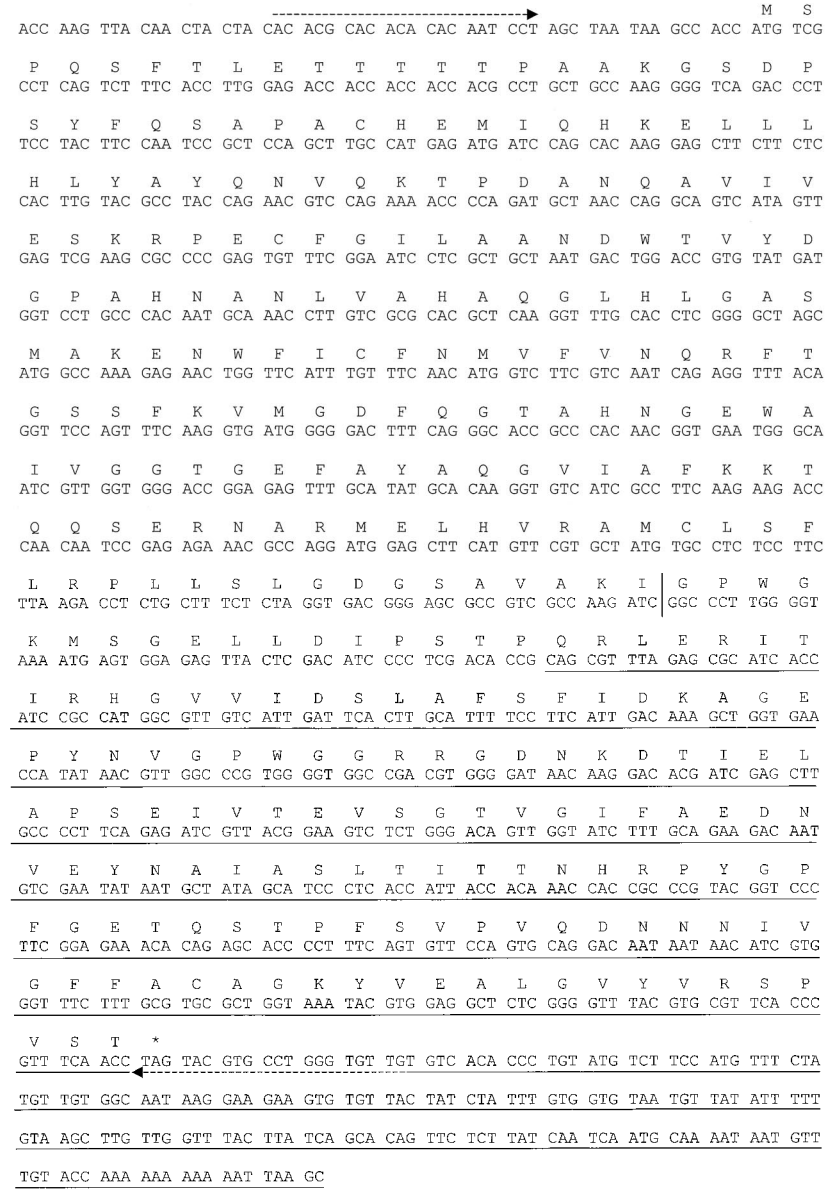


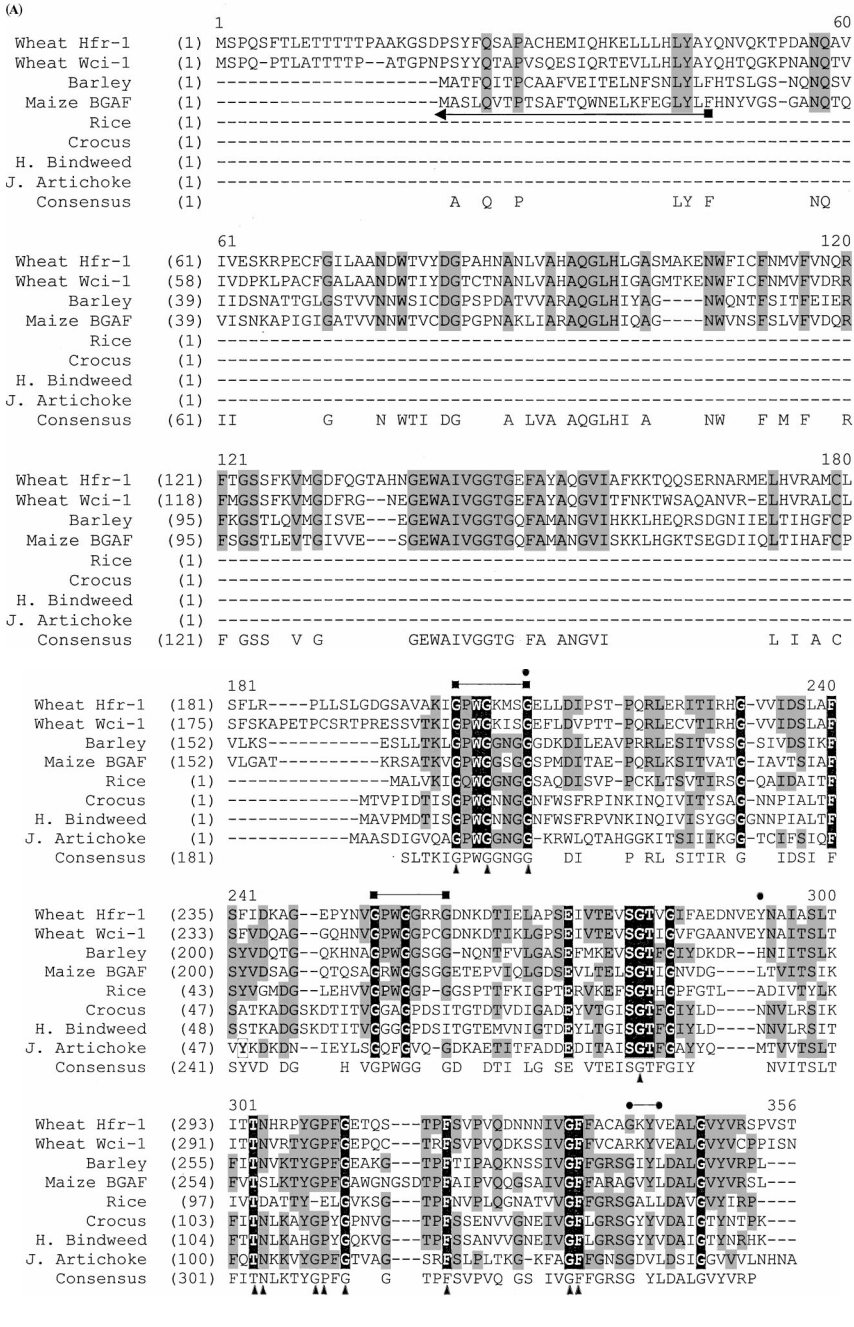
FIG. 3. Nucleotide and deduced amino acid sequence of *Hfr-1*. The near full-length nucleotide sequence of the *Hfr-1* cDNA is shown with the deduced amino acid sequence above each codon. The nucleotide coordinates are indicated to the left. The termination codon is indicated with an asterisk. The sequences corresponding to the forward and reverse primers for the clone UPW1Hfr1a are indicated with dashed arrows. The sequence of the clone UPW1Hfr1b, which was used for blot hybridizations, is underlined. The N-terminal and C-terminal amino acid domains are separated by a vertical line.

protein sequence. Mannose-binding jacalin-like lectins, the class most similar to the C-terminus of the Hfr-1 deduced amino acid sequence, contain a fair amount of sequence diversity. However, all proteins that have been shown to exhibit mannose-binding lectin activity have 12 invariant amino acid residues, also invariant in the Hfr-1 sequence (Figure 4a), that are necessary for the integrity of the β prism fold (Bourne et al., 1999). Some of the residues known to exhibit mannose-binding lectin activity, as well as the residues of the β-glucosidase-binding sites of BGAF, are highly conserved across this group of related proteins (Figure 4a).

Elements of the N-terminal domain of Hfr-1 are found in several *Arabidopsis* defense-response genes that are identified in database searches. Part of this N-terminal domain is cleaved from BGAF to produce the active protein (Blanchard et al., 2001). Similarly, a small polypeptide is cleaved from the N-terminal end of the mannose-binding lectins to produce the mature proteins (Zhang et al., 2000) (Figure 4a). These cleavage sites are not classical signal peptide sites, but they were determined experimentally. It is unknown whether, like related proteins, the N-terminal domain of the Hfr-1 sequence is cleaved in vivo. However, a comparison of the C-terminal portion of the deduced Hfr-1 protein sequence, starting at amino acid 203 (Figure 4a), with the β prism region of known mannose-binding jacalin-like lectins revealed that identity varied from 41% with jackfruit (Genbank number AAA32679) to 33% with Jerusalem artichoke (Genbank number AAD11577) and 30% with hedge bindweed (Genbank number AAC49564).

DISCUSSION

Because wheat and the Hessian fly interact in a gene-for-gene manner, the response of a given plant genotype may result in either a compatible or an incompatible interaction, depending on the genotype of the infesting larvae. Likewise, a single Hessian fly genotype may be virulent on some lines of wheat and avirulent on others, depending on the genotype of the host plant. Thus, the outcome of an interaction is determined by the ability of the plant to recognize and defend against the invader, coupled with the insect's effectiveness in avoiding recognition. The success and failure of larval recognition, mediated by the *H9* resistance gene in these experiments, resulted in up- and down-regulation of different sets of genes (C. C. Collier and C. E. Williams, unpublished results) and markedly different plant phenotypes. The plant phenotype resulting from the compatible interaction was distinct, characterized by systemic stunting of the leaves. However, during incompatible interactions, typically no visual phenotypic change in the plant is noted. It has been unclear whether the resistant wheat plant passively blocks the effect of larval secretions, leaving the insect to die of starvation, or actively mounts a local defense. Obviation of resistance during mixed infestations (Grover et al., 1989) suggests


(A)
Wheat Hfr-1
Wheat Wci-1
Barley
Maize BGAF
Rice
Crocus
H. Bindweed
J. Artichoke
Consensus
(B)
N-terminal
C-terminal
1
44
179
195
286
348
356
Disease Resistance Response
Mannose-Binding Jacalin-Like Lectin
Myrosinase-Binding

that systemic induction of toxin production is not a major component of the plant's response to avirulent larvae. However, the increased expression of the *Hfr-1* gene in leaf blades demonstrated that recognition of avirulent first-instar larvae initiated an active response that may involve local and systemic defense mechanisms. The increased *Hfr-1* gene expression during the incompatible interaction corresponded to the period when first-instar larvae were attempting to feed. *Hfr-1* mRNA levels returned to low expression at a time when avirulent larvae appeared dead.

This study focused on wheat–Hessian fly interactions during the first 136 hr of infestation, which corresponded to the period when first-instar larvae were injecting substances into the plant and attempting to establish feeding sites. During this period, *Hfr-1* mRNA, which is similar to the mRNA sequence for a *BGAF* gene from maize, increased in abundance. The maize BGAF protein is capable of forming large complexes with β-glucosidases. Since BGAF does not interfere with β-glucosidase activity, it may protect an endogenous plant β-glucosidase that activates 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a major defense compound active against insect pests of maize and wheat (Gianoli and Neimeyer, 1998), from proteolytic enzymes in larval saliva (Blanchard et al., 2001). Genes responsible for accumulation of DIMBOA are located on wheat chromosome 4A (Neimeyer and Jerez, 1997), the same chromosome that includes one of the two copies of *Hfr-1* in Chinese Spring wheat (K. M. Devos and C. E. Williams, unpublished results).

Saliva from several insects has been shown to contain β-glucosidase (Mattiacci et al., 1995; Miles, 1999). Although it is not a major component, β-glucosidase is detectable in the saliva of virulent second-instar Hessian fly larvae

FIG. 4. Sequence conservation. (A) Alignment of the deduced amino acid sequences for *Hfr-1* and sequences encoded by related genes (Genbank numbers: wheat Wci-1, AAC49284.1; barley JA-induced gene, AAA87042.1; maize BGAF, AAF71261.2; rice lectin, BAB19404.1; crocus lectin, AAG10403.1; hedge bindweed lectin, AAC49564.1; Jerusalem artichoke lectin, AAD11577.1). Multiple sequence alignment was constructed with ClustalW. The first four sequences are substantially longer than the last four, which are known to function as mannose-binding jacalin-like lectins. Residues that are identical in at least four of the eight sequences are highlighted in gray. Residues that are invariant are highlighted in black. The arrow with a black square indicates the N-terminal sequence that is cleaved to activate the BGAF protein. The two bars with black squares indicate the regions of BGAF that bind β-glucosidase. These sites are partially conserved in the other sequences. Black circles indicate regions of the four jacalin-like lectins that bind mannose. The 12 amino acid positions indicated by black triangles are conserved in all mannose-binding lectins (modified from Figure 1 in Bourne et al., 1999). (B) Domains of the Hfr-1 deduced amino acid sequence. The N-terminal region that shares sequence similarity with disease resistance response genes spans amino acids 44–179. The C-terminal mannose-binding jacalin-like lectin domain spans amino acids 195–348 and the region that is similar to myrosinase-binding proteins spans amino acids 286–348.

and may be more abundant in the first-instar larval saliva that is injected into plant tissues. Because of greatly increased larval size over first-instars, the contents of saliva from virulent second-instar Hessian fly larval only have been analyzed (Grover et al., 1988). However, it is the first-instar rather than the second-instar larvae that pierce and inject substances into plant tissues. Second instars no longer need to or are capable of piercing, because in order for the larvae to survive, the relevant changes in plant cell permeability had to be initiated through a compatible interaction during the first instar. β-Glucosidases activate stored cytokinins (Brzobohaty et al., 1993) and defense molecules such as salicylic acid (Wallner and Walker, 1975), which is an inducer of *Hfr-1* expression (J. M. Meyer and C. E. Williams, unpublished results). Because they also hydrolyze cell wall polysaccharides (Wallner and Walker, 1975), β-glucosidases from larval saliva may contribute to partial cell wall degradation and permeability in the wheat–Hessian fly interaction. Similar to the silverleaf whitefly, which may produce elicitors in nymphs but not in adults for the *SLW1* and *SLW3* genes of squash plants (van de Ven et al., 2000), components of Hessian fly larval saliva may vary according to feeding strategy at different instars. Alternatively, a larval genotype may vary the components of its saliva, depending on whether the plant is resistant or susceptible (once the plant's defense is compromised), much like hemipteran salivary protein profiles that can change in response to different host plant substrates (Habibi et al., 2001). Thus, the *Hfr-1* gene sequence is similar to a gene encoding a protein that agglutinates cell-damaging β-glucosidases. However, the Hfr-1 deduced amino acid sequence, as well as that of Wci-1 and BGAF, share so many structural elements and active sites with lectins that their *in vivo* functions may be more closely aligned with lectin activity than with agglutination of β-glucosidases.

The sequence similarity between the mannose-binding jacalin-like lectins and the C-terminal domain of the deduced amino acid sequences of Hfr-1, plus the wheat Wci-1, maize BGAF and barley sequences shown in Figure 4a, strongly suggested that all belong to the same superfamily of lectins. Although Hfr-1, Wci-1, BGAF, and the barley sequences contained N-terminal regions that are not found in the sequences that are known to function as lectins, much of the C-terminal sequence corresponding to the β prism fold was conserved. The β prism sequence is divergent among active lectins, allowing agglutination of a wide variety of glycoproteins. However, 12 amino acids, essential for proper folding, are conserved among all mannose-binding lectins (Bourne et al., 1999). All 12 of these amino acids were present in Hfr-1, Wci-1, and the barley sequences, and only one of the 12 was missing from BGAF. Most plant lectins target foreign glycans rather than endogenous plant carbohydrates (Peumans et al., 2000), and several are implicated in resistance to insects. The mannose-binding snowdrop lectin binds to the mid-gut of the brown planthopper and the green leafhopper (Foissac et al., 2000), preventing nutrient absorption. In addition, snowdrop lectin acts as a feeding deterrent for these insects. Experiments are underway to determine whether

the protein encoded by *Hfr-1* has lectin activity. No discrete function has been assigned to molecules sharing the domain that is part of the N-terminal sequence of Hfr-1 and the other lectin-like proteins from monocots. Further experimentation will determine whether this domain contains cleavage sites similar to those needed for production of the mature forms of the BGAF protein and the mannose-binding lectins (Blanchard et al., 2001; Zhang et al., 2000).

In conclusion, the rapid increase in expression of *Hfr-1* mRNA was similar to the expression profiles of plant defense–response genes during incompatible interactions between microbial pathogens and their monocot hosts: wheat (Rebmann et al., 1991; Schaffrath et al., 1997; Molina et al., 1999), barley (Hahn et al., 1993; Mouradov et al., 1994), oat (Lin et al., 1996), and maize (Morris et al., 1998). Related defense–response genes, such as *PR-1*, that are characteristic of responses to microbial attack are also induced during interactions involving *Arabidopsis* and phloem-feeding aphids (Moran and Thompson, 2001). Furthermore, Russian wheat aphids feeding on wheat cause rapid increases in expression of the PR proteins chitinase, peroxidase, and β-glucanases (van der Westhuizen and Pretorius, 1998; van der Westhuizen et al., 1998). Thus, responses to diverse organisms share common elements. Defense–response genes are not limited to a standard set of genes or pathways, however. Four pathogen-response signaling pathways are prominent in addition to other less well-characterized pathways that lead to novel gene expression (reviewed in Walling, 2000). Some elicitors activate novel genes involved in systemic species-specific responses, such as genes responding to whitefly feeding on squash (van de Ven et al., 2000). Although induction of an *Hfr-1*-like gene during plant defense has not been reported previously, related jasmonate-induced genes have been noted in barley (Chaudhry et al., 1994). The timing of *Hfr-1* expression plus its sequence similarity to lectins and other genes implicated in insect resistance suggested that it may play a role in defending wheat against infestation by Hessian fly larvae.

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POLYMORPHISM IN MAJOR URINARY PROTEINS: MOLECULAR HETEROGENEITY IN A WILD MOUSE POPULATION

ROBERT J. BEYNON,[1,*] CHRISTINA VEGGERBY,[1] CAROLINE E. PAYNE,[2] DUNCAN H. L. ROBERTSON,[1] SIMON J. GASKELL,[3] RICK E. HUMPHRIES,[2] and JANE L. HURST,[2]

[1]*Protein Function Group*

[2]*Animal Behaviour Group*
Faculty of Veterinary Science
University of Liverpool
PO Box 147
Liverpool, L69 3BX, United Kingdom

[3]*Michael Barber Centre for Mass Spectrometry*
UMIST
PO Box 88
Manchester M60 1QD, United Kingdom

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Abstract—Major urinary proteins (MUPs) are present in high levels in the urine of mice, and the specific profile of MUPs varies considerably among wild-caught individuals. We have conducted a detailed study of the polymorphic variation within a geographically constrained island population, analyzing the MUP heterogeneity by isoelectric focusing and analytical ion exchange chromatography. Several MUPs were purified in sufficient quantities for analysis by electrospray ionization mass spectrometry and MALDI-TOF mass spectrometry of endopeptidase Lys-C peptide maps. The results of such analyses permitted the identification of three new MUP allelic variants. In each of these proteins, the sites of variation were located to a restricted segment of the polypeptide chain, projecting to a patch on the surface of the protein, and connected to the central lipocalin calyx through the polypeptide backbone. The restriction of the polymorphic variation to one segment of the polypeptide may be of functional significance, either in the modulation of ligand release or in communication of individuality signals within urinary scent marks.

Key Words—Major urinary proteins, mouse, MALDI-TOF mass spectrometry, protein purification, isoelectric focusing.

* To whom correspondence should be addressed. E-mail: r.beynon@liv.ac.uk

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INTRODUCTION

The urine of house mice (*Mus domesticus*) contains a high concentration of protein, virtually all of which consists of members of an 18- to 19-kDa protein family termed "major urinary proteins," (MUPs). These MUPs are lipocalins (Flower et al., 1993), characterized by an archetypical eight-stranded β-barrel (Bocskei et al., 1992; Lucke et al., 1999; Zidek et al., 1999), enclosing a hydrophobic cavity. A generic role of the lipocalin family is the binding of small hydrophobic ligands (Flower et al., 1993, 2000), and in this respect, the MUPs are typical. Thus far, five endogenous MUP ligands have been reported; 2-*sec*-butyl-4,5-dihydrothiazole, 3,4-dehydro-*exo*-brevicomin, α- and β-farnesene, and 6-hydroxy-6-methyl-3-heptanone (Bocskei et al., 1992; Bacchini et al., 1992; Robertson et al., 1993; Novotny et al., 1999; Timm et al., 2001). All of these ligands are semiochemicals and are known to elicit primer pheromonal effects (Jemiolo et al., 1985, 1986).

Until recently, the necessity of protein binding for the activity of these ligands has not been understood, but we have provided direct behavioral and biochemical evidence that MUPs slow the release of semiochemicals from deposited urine samples (Hurst et al., 1998; Beynon et al., 1999). However, a simple role of ligand binding and release does not explain the observation that MUPs are encoded by a large, multigene complex and that urine from individual mice contains several MUPs, each a discrete gene product. Although there is some variation in ligand binding capability between different MUPs (Marie et al., 2001; Robertson et al., 2001), differential ligand release kinetics seems inadequate as an explanation for MUP diversity, since many MUPs show similar ligand binding properties. We have shown that protein fractions, depleted of natural volatile ligands by aging or by competitive displacement, elicit a strong countermarking response (Humphries et al., 1999) and, thus, MUPs or nonvolatile MUP–ligand complexes communicate chemical information in their own right. This is consistent with the hypothesis that proteins act as a stable signal of an animal's presence and are the medium through which scent marks evince the presence of a different individual. The growing evidence for other roles of MUPs in chemical signaling is also relevant. Brennan et al. (1999) suggested that these proteins provide one component of the strain recognition signal, and Mucignat-Caretta et al. (1995) showed that MUPs interact with receptors in the vomeronasal organ to exert an effect on puberty acceleration in females, even without the ligands. We have suggested that the MUPs, possibly in combination with ligands, have the ability to be used to communicate individuality within a mouse population (Beynon et al., 1999), and we recently provided direct evidence to support this hypothesis (Hurst et al., 2001).

Most of the information relating to MUPs has derived from studies of the proteins from relatively few inbred mouse strains (Robertson et al., 1996 and references therein). Since each strain is genetically homogeneous, it must be considered as a single snapshot of the wild mouse genome and, as such, yields little

information on the extent of genetic diversity in the wild house mouse. Accordingly, we have embarked on a study of the MUPs from wild mice. Our initial screen of a small number of wild mice from several sites in the UK yielded clear evidence of a hitherto unsuspected degree of polymorphism (Robertson et al., 1997; Pes et al., 1999; Payne et al., 2001). In this study, we have focused on MUP diversity from a single island population of mice to establish further the degree of polymorphism, particularly in a geographically and, therefore, genetically constrained population. The Isle of May (54 ha) in the Firth of Forth, approx. 10 km from the Scottish mainland, is uninhabited (except for wardens and a small number of researchers during the summer months) and contains a population of between a few hundred and a few thousand mice that vary cyclically over the year (Triggs, 1991). Indeed, until the introduction of a small number of mice from another island in 1982, the May mice were homozygous for over 70 loci, including the major histocompatibility complex (Berry and Peters, 1977; Berry et al., 1991). Subsequently, the new alleles penetrated rapidly through the population and reached equilibrium within three years (Figueroa et al., 1986; Jones et al., 1995). This rapid penetration may have been aided by the lack of territorial division in this population, leading to considerable genetic mixing. In contrast to the strongly territorial behavior normally seen among house mice living commensally in the built environment, competitive behavior appears to be virtually absent among feral males from the Isle of May (Gray and Hurst, 1998), and individual home ranges overlap extensively (Hurst, unpublished data). However, even within such an isolated and freely mixing population, where overall genetic variation is low, we should expect relatively high variation among individuals in cues that are used for individual recognition and perhaps also to recognize close relatives. Further identification of the sites of variation between the MUPs expressed by different individuals may indicate whether variation lies largely at the ligand-binding site (suggesting that individuality may be signaled through differential binding and release of volatile ligands) or lies on the surface of the protein for direct receptor-mediated detection. This paper reports detailed characterization of the MUPs expressed by different individuals within the Isle of May population and provides evidence for several new allelic variants not previously encountered.

METHODS AND MATERIALS

Chemicals. Acetonitrile, triflouroacetic acid, and methanol, all HiPerSolv grade, were obtained from BDH (Poole, England). Formic acid, NaCl, β-mercaptoethanol, EDTA, Tris, and trichloroacetic acid, all AnalaR grade, were obtained from BDH. Peptides (des-Arg bradykinin, neurotensin, ACTH, and insulin B-chain), horse heart myoglobin, Coomassie blue, Bistris, and α-cyano-4-hydroxycinnamic acid were obtained from Sigma (Poole, England). The Coomassie

Protein Plus assay including BSA standard was obtained from Pierce & Warriner, Chester, UK. The endoprotease Lys-C (sequencing grade) was obtained from Roche (Lewes, England).

Mice and Urine Collection. Male wild mice were captured from the population living on the Isle of May. The mice were housed individually in cages (30 × 13 × 12 cm) on peat substrate with shredded paper nest material and ad libitum food (Teklad Mouse/Rat diet 9609; Banting and Kingman, Hull, UK, and wheat grain) and water. Urine, voided in response to the mouse being held by the scruff of the neck, was collected into 1.5-ml Eppendorf tubes and frozen immediately. Many samples were no greater than 10–20 μl, which precluded exhaustive analysis.

Isoelectric Focusing (IEF). Isoelectric focusing was performed using Immobiline dryplates pH 4.2–4.9 (AP Biotech) on the Multiphor flatbed system cooled to 10°C with a Multitemp III thermostatic circulator (AP Biotech). Samples were diluted 1:10 in water, and 5 μl was loaded onto the gel at 200 V, 5 mA, and 15 W for 200 vh. Sample applicators were removed and the gel run at 3500 V, 5 mA, and 15 W for 14.8 kVh. The gel was stained with Coomassie Brilliant Blue in a Hoefer automated gel stainer.

Ion Exchange Chromatography (IEC). Urinary proteins were purified by high-resolution anion exchange chromatography on the SMART chromatography platform (AP Biotech). Prior to chromatography, 10 μl urine was desalted on micro Sephadex G25 spin columns and filtered through a 0.22-μm filter. Protein concentration of the desalted urine was measured (Coomassie Protein Plus assay). Fifteen micrograms of urinary protein was applied to a MiniQ PC 3.2/3 column (240 μl bed volume, Pharmacia) equilibrated with 20 mM Bistris buffer, pH 5.5, at a flow rate of 120 μl/min. Following sample application, the column was washed with 5 column volumes of 20 mM Bistris buffer, pH 5.5. Bound protein was eluted with a linear gradient of NaCl from 0 to 150 mM in the same buffer in 20 column volumes. Protein peaks, identified by absorbance at 280 nm, were collected. Peak fraction sizes were typically 60 μl, containing 1–3 μg of protein.

Electrospray Ionization Mass Spectrometry (ESI-MS). Peak fractions from IEC were desalted into 0.2% (v/v) formic acid on Microcon-10 concentrators (Millipore, Watford, England). Desalted samples were diluted 1:10 in 50% (v/v) acetonitrile–0.1% (v/v) formic acid. All analyses were performed on a VG Quattro mass spectrometer, upgraded to Quattro-II specifications (Micromass, Manchester, UK), fitted with an electrospray ionization source. Samples were introduced into the mass spectrometer as a continuous infusion at 5 μl/min. Raw data, gathered between *m/z* 700 and 1400, were subsequently refined and transformed to a true mass scale using Maximum Entropy software. Raw data were processed between 18,400 and 19,000 Da. The instrument was calibrated using a 2 pmol/μl solution of horse heart myoglobin in 50% (v/v) acetonitrile–0.1% (v/v) formic acid run after each sample. The calibration spectrum also served to determine the peak width parameter (0.75 Da) during Maximum Entropy processing.

Lys-C Digestion. The proteins in peak fractions from ion exchange chromatography were denatured in 4 M guanidinium thiocyanate for 1 hr and desalted into Lys-C digestion buffer (25 mM Tris, 1 mM EDTA, 2 mM β-mercaptoethanol, pH 8.5) on Microcon-10 concentrators. Sequencing grade Lys-C was added in a 1:100 (w/w) ratio. Digestion took place overnight at 37°C, and the reaction was stopped by the addition of 1 μl formic acid. A reference peptide digest of uMUP-I from BALB/C was prepared by digesting purified uMUP-I (IEC on the SMART system) as described above.

MALDI-TOF MS. Peptides were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (PE Biosystems Voyager Elite, Warrington, UK), equipped with a 2-m linear and a 3-m reflectron flight mode and a delayed extraction function. A pulsed nitrogen laser emitting at 337 nm was used as a desorption/ionization source. In all experiments, mass spectrometry was performed in reflectron mode with positive ion detection. Instrument parameters were as follows: accelerating voltage 20 kV, grid voltage 73.5%, guide wire voltage 0.05%, and pulse delay time 175 nsec. Laser energy was ca. 1500. External mass calibration was performed with a mixture of des-Arg bradykinin, neurotensin, adrenocorticotrophic hormone, and insulin β-chain (50 nM each in 50% acetonitrile–0.1% triflouroacetic acid). To 1 μl of digested protein or peptide calibrants, 1 μl of α-cyano-4-hydroxycinnamic acid (saturated in 50% acetonitrile–0.1% triflouroacetic acid) was added, and 1 μl of the mixture was spotted onto the target. Signal-averaged mass spectra for 80–256 laser shots were collected, and data were analyzed using the PE Biosystems GRAMS/386 software.

RESULTS AND DISCUSSION

We have previously examined in detail the MUP allelomorphs from two inbred laboratory strains of mice: BALB/C and C57BL/6J (Robertson et al., 1996) in which we were able to reconcile urinary protein expression with known cDNA sequences and provide evidence, purely from protein analysis, for new gene products for which no cDNA sequences were known. These inbred strains, BALB/C and C57BL/6J, can be construed as two snapshots of the wild mouse genome, and our data provided evidence for at least 14 proteins potentially or actually expressed in these two strains. Further work on limited numbers of wild-caught specimens yielded clear evidence for remarkable diversity in MUP profiles (Pes et al., 1999; Payne et al., 2001), suggesting many as yet uncharacterized variants of these proteins. Accordingly, we embarked upon a systematic study of MUP expression in mice sampled from a closed, island population. In addition to yielding further information on MUP polymorphism, these samples were informative in studies on MUP pattern complexity within a closed but natural population, the results of which will be reported elsewhere. In this paper, we describe the isolation and characterization of the most abundant MUPs found in this population.

Survey of MUP Expression in Isle of May Mouse Urine Samples. Charge and exact mass are valuable parameters in the screening for novel MUPs (Robertson et al., 1997). Isle of May mouse urine samples separated on linear SDS–polyacrylamide gel electrophoresis showed a strong protein band at 18 kDa and little or no other protein constituents. When electroblotted to nitrocellulose, the 18 kDa band reacted strongly with an anti-MUP polyclonal antiserum (results not shown). Accordingly, we assumed that the detectable proteins in a mouse urine sample were predominantly MUPs, an assumption that has been repeatedly validated. For rapid initial screening of samples derived from Isle of May mice, urinary proteins from 17 wild caught animals were analyzed by IEF using the method described in Payne et al. (2001) (Figure 1). Whereas all individuals from any inbred mouse strain would have completely identical profiles in IEF (a BALB/C sample is included in the figure for comparison), the profiles of these 17 wild mice differed among individuals, although there was evidence of widespread expression of common protein subtypes. Between four and 10 bands were expressed in each of the Isle of May urine samples, but there was considerable overlap of protein expression in these individuals—a total of only 11 different bands could be discerned. By comparison with analyses of urine samples from mainland-caught mice, the patterns were much more similar among individuals, which may reflect the limited number of founder animals, or genetic bottlenecks due to poor overwinter survival

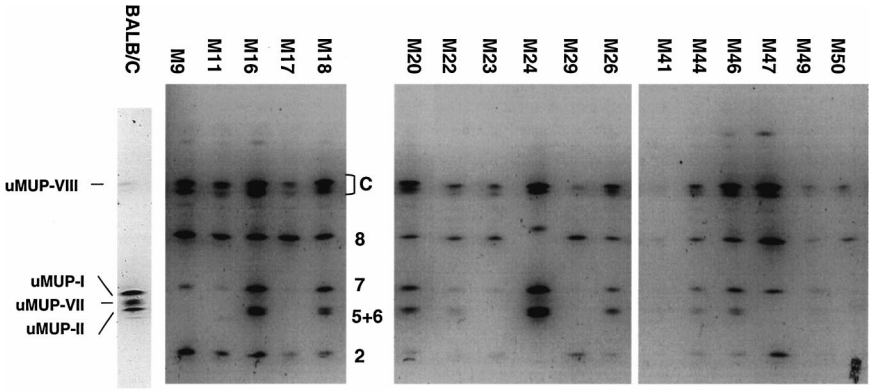


FIG. 1. Isoelectric focusing of MUPs from 17 wild mice from the Isle of May. Isoelectric focusing was performed using Immobiline dry plates, pH 4.2–4.9 (AP Biotech) on the Multiphor flatbed system (AP Biotech). Samples were diluted $1 + 9$ in water, 5 μl was applied and the gel was run at 3500 V, 5 mA, and 15 W for 14.8 kVh. A sample of urine from a BALB/C inbred mouse was included for comparison. Bands identified by analysis of proteins purified by ion exchange chromatography are labeled for cross-reference elsewhere in the paper. Known MUP variants from BALB/C urine are labelled with the standard nomenclature.

that is a feature of this geographically constrained population (Berry and Peters, 1977; Berry et al., 1991; Jones et al., 1995; Payne et al., 2001).

Chromatographic Separation of MUPs from Isle of May Mice. For further characterization of the proteins, MUPs from the wild-caught May mice and from inbred BALB/C and C57BL/6J mice were purified by high-resolution IEC (MiniQ column, SMART chromatography platform). A representative elution profile for one Isle of May mouse (M22), and the profiles from the well-characterized inbred strains BALB/C and C57BL/6J are given in Figure 2. A low loading (15 μg of protein) was necessary for reproducible and high-resolution separation of these mixtures of very similar proteins. The M22 mouse sample contained 11 discernible protein peaks, all eluting between 2.8 and 4.9 ml, thus displaying greater complexity than inbred mice (seven and five peaks for BALB/C and C57BL/6J, respectively). The first six and last three peaks were well separated, whereas the proteins at $V_e = 3.76$ ml and 3.86 ml overlapped and probably contained more than one protein each, as both were irregularly shaped with shoulders. The large, complex peak between $V_e = 3.76$ and 3.86 ml is a persistent feature of urine samples from Isle of May mice but is not present in either inbred strain. When comparing the peak elution volumes (V_e) to those of the inbred mice proteins, some overlap was found. Five peaks ($V_e = 2.97$, 3.38, 3.50, 3.76, and 4.82 ml) were matched by similar peaks in BALB/C ($V_e = 2.98$, 3.40, 3.49, 3.73, and 4.84 ml), and four May peaks ($V_e = 3.50$, 3.63, 3.76, and 4.82 ml) matched to equivalents in C57BL/6J ($V_e = 3.48$, 3.61, 3.77, and 4.83 ml), indicating the presence of proteins of similar charge in the three different samples.

Ion exchange chromatography identified more charge variants than IEF, although for the major proteins, the results of the two analyses correlated well. Chromatographically purified MUPs from M18 and from BALB/C were analyzed by IEF, and the peaks that could be identified are labeled in Figure 1. The BALB/C MUPs had been previously identified and were labeled according to the proposed nomenclature (Robertson et al., 1997). When the remainder of the Isle of May samples were analyzed by high-resolution IEC, similarly complex elution profiles were obtained, with 6–11 peaks expressed in each sample. These elution traces are not shown, but representative profiles are summarized in Figure 3. Because a constant amount of protein was applied to the column for each individual urine sample, comparison of the expression of different peaks was possible. The chromatographic traces revealed differences between almost every animal. Two proteins were found in a single individual: peak 1 was seen only in M15 and peak 9 was only expressed in M24. The V_e of these two proteins differed from the nearest groups by more than 2 SD and are, therefore, likely to be separate proteins. At the other extreme, several peaks (2, 3, 6, 7, 8, 11) were present in over 50% of the samples analyzed, the cluster proteins were found in all mice tested, and peaks 2, 7, and 8 were observed in all but one individual. Many individuals share the major proteins (2, 3, 5, 6, 7, 8, cluster, 11). Of the major proteins, peaks 5 and 6 demonstrated the

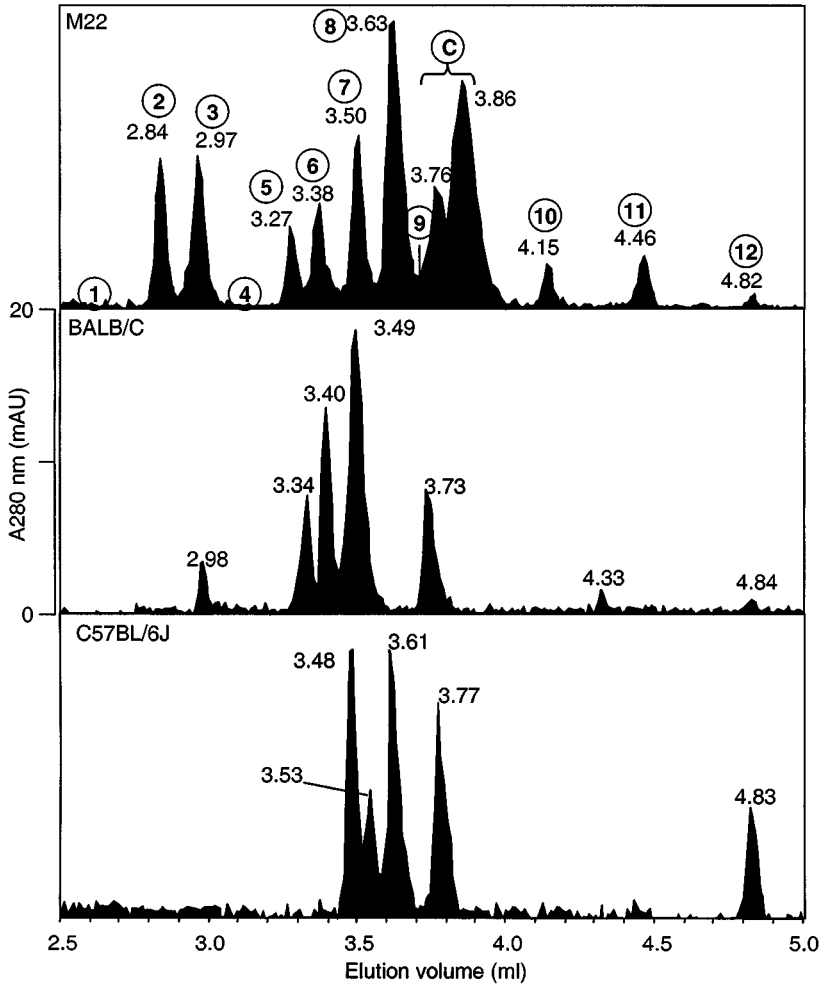


FIG. 2. Purification of MUPs from the M22 wild mouse. Urine samples from Isle of May mouse M22, BALB/C, and C57BL/6J were desalted into 20 mM Bistris buffer, pH 5.5, and 15 μg of protein was applied to a MiniQ column ($V_t = 240$ μl). Bound proteins were eluted with a linear salt gradient from 0 to 150 mM NaCl in 20 column volumes of the same buffer. The black areas define $A_{280\,nm}$. All three panels are displayed at the same scale, and each peak is annotated with the peak number and elution volume in milliliters.

greatest variability among individuals, but there was more pronounced variability in expression of the minor components. At present, we have no knowledge of the ability of mice to perceive differences in the relative concentrations of MUPs in a urine sample, and the significance of such variability is unknown.

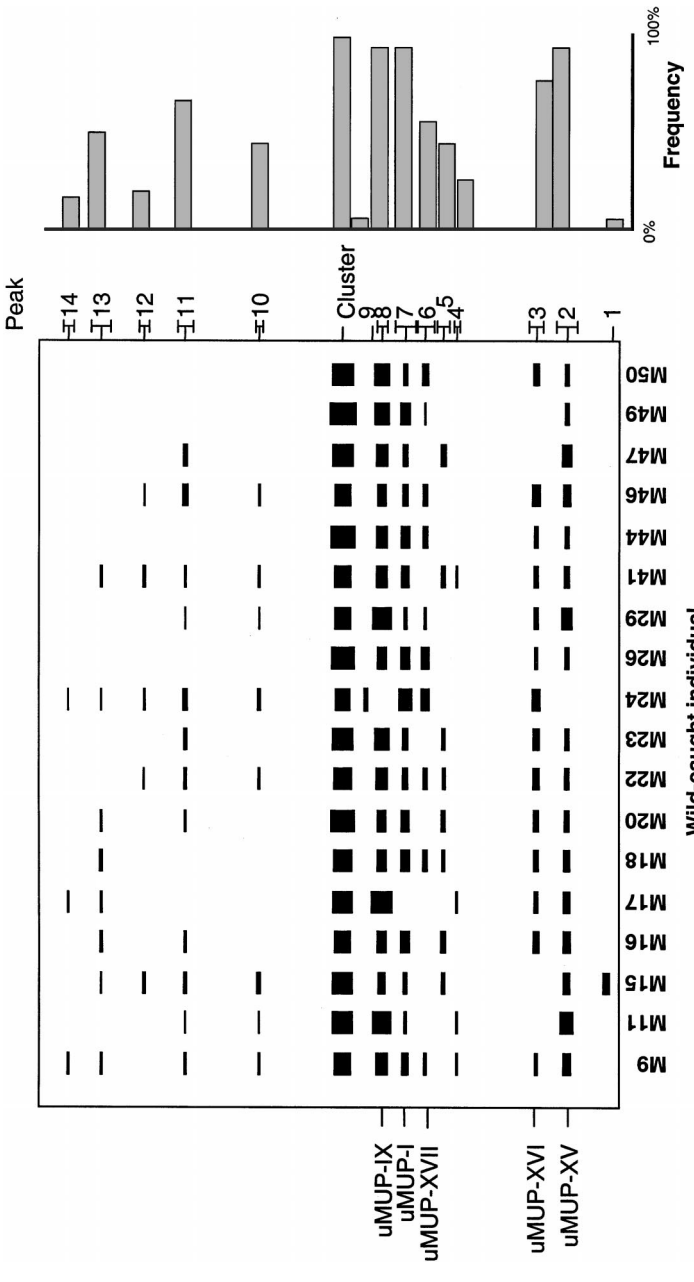


FIG. 3. Summary of IEC of MUPs from wild-caught mice from the Isle of May. MUPs from 18 Isle of May mice were purified by ion exchange chromatography on the SMART system, as described for M22 (see Figure 2 legend). Peaks from the different Isle of May mice were grouped according to V_e and are represented as bands at the average peak elution volume of the group. The width of the band represents the relative peak area (calculated as a percentage of the total peak area). The error bars to the right of the figure represent the mean $V_e \pm 2$ SD of each protein peak. The standard deviations of the elution volumes in the groups varied from 0.005 to 0.020 ml, which is comparable to that achieved from multiple runs of BALB/C (S.D. 0.01–0.02 ml for peaks 1–7, $N = 6$). The peaks between 3.69 and 3.88 ml are not completely resolved in any of the samples and are, therefore, presented as one cluster. Protein from groups annotated with an uMUP name are those that have been previously identified in inbred mouse strains or which were characterized as novel proteins by ESI-MS and peptide fingerprinting. The frequency of expression of each of the peaks is summarized to the right of the figure.

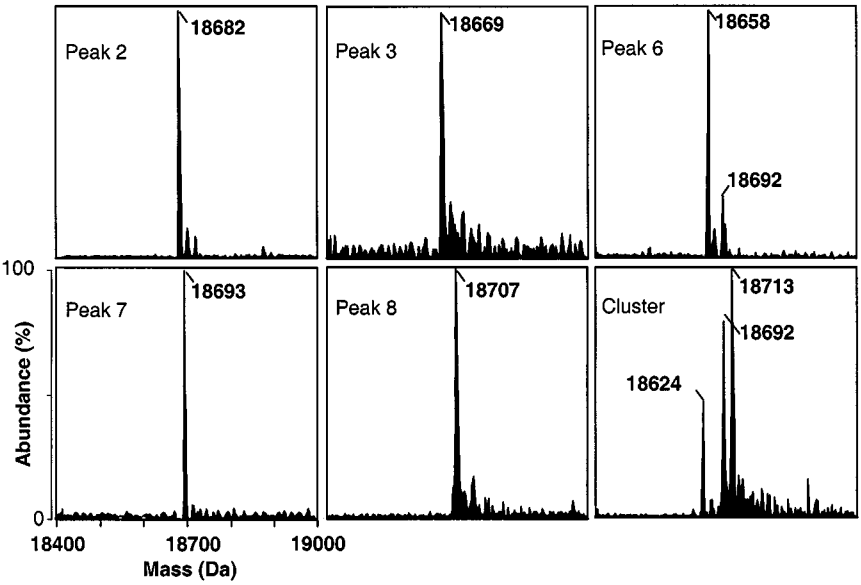


FIG. 4. Electrospray mass spectrometry analysis of purified M22 MUPs. IEC purified M22 urinary proteins were desalted into 0.2% formic acid and analyzed on a VG Quattro mass spectrometer, upgraded to Quattro-II specifications, fitted with an electrospray ionization source. Raw data, gathered between m/z 700 and 1400, were subsequently refined and transformed to a true mass scale using Maximum Entropy software. Raw data were processed between 18,400 and 19,000 Da. All six panels are displayed at the same scale.

Electrospray Ionization Mass Spectrometry of Purified Intact Proteins. To establish that the previously unseen species on IEF and IEC were new MUPs, the more abundant proteins were isolated by IEC of urine samples derived from individuals M9, M16, M18, M22, and M41. The major peaks (2, 3, 6, 7, 8, and the cluster mixture) were separately desalted into 0.2% formic acid and analyzed by electrospray ionization mass spectrometry (Figure 4). Each of the five main chromatographic peaks contained a predominant species, as assessed by accurate mass measurement (peak 6 contained a trace of a protein of mass 18,692 Da, possibly slight contamination from peak 7). The same chromatographic peaks from all five mice contained proteins of identical masses (mean $\pm$ SD): peak 2 was $18{,}682 \pm 0.3$ Da ($N = 5$), peak 3 was 18,668 Da (mean, $N = 2$), peak 6 was $18{,}658 \pm 0.9$ Da ($N = 3$), peak 7 was $18{,}693 \pm 0.5$ Da ($N = 5$), and peak 8 was $18{,}707 \pm 0.4$ Da ($N = 5$). From each mouse, the cluster contained protein species of three or four distinct masses, of which two (18,692 and 18,713 Da) had previously been observed in MUPs isolated from inbred animals (Robertson et al., 1997), and which could be reconciled to known cDNA sequences. It has not

proved possible to resolve the individual proteins in this cluster, and they have not been studied further.

Two of the proteins in the chromatographically pure peaks exhibited masses that were identical to the masses of proteins expressed in the urine of inbred mice (peak 7, 18,693 Da and peak 8, 18,707 Da) and that eluted in the same positions on IEC. The 18,693-Da peaks from BALB/C (uMUP-I) and Isle of May mice (peak 7) eluted at 3.49 ml and 3.50 ± 0.02 ml (mean $\pm$ SD), respectively, and the 18,707 Da peaks from C57BL/6J (uMUP-IX) and Isle of May mice (peak 8) eluted at 3.61 ml and 3.62 ± 0.01 ml, respectively. These proteins were, thus, indistinguishable on the basis of mass and charge, and we conjectured that these are the products of the same genes (subsequently proven by peptide mass fingerprinting, below).

Knowledge of the overall charge (isoelectric focusing and ion exchange chromatography) and intact protein mass are valuable parameters for the identification of new polymorphic variants of the proteins. However, these global parameters do not provide information on the site of protein sequence variation nor, indeed, do they exclude the possibility that these variants could be derived through posttranslational modifications. To characterize the new proteins further, we used MALDI-TOF peptide mass fingerprinting. The purified proteins were denatured and digested to limit peptides with the endopeptidase Lys-C, which cleaves specifically at the carboxyl side of lysyl residues. The resultant limit peptide mix was analyzed by MALDI-TOF mass spectrometry (Figure 5). Endopeptidase Lys-C is particularly valuable for the analysis of MUPs, since the limit digestion products (labeled L1 to L11 for the uMUP-I endopeptidase Lys-C limit peptides) were mostly large enough to be displaced from the chemical (mainly matrix) noise in the MALDI-TOF spectrum below 800 Da. All of the limit peptides, with the exception the two tripeptides L3 and L6, were observed and 96% (156/162) of the sequence was, thus, accounted for. As the protein sequence for uMUP-I was known (Genbank accession M16355), the peptide masses could be matched to that of the theoretical digest pattern for this protein, used as a reference for peptide mapping (Figure 6).

When comparing the peptide masses found for the five Isle of May MUPs to those of the BALB/C uMUP-I peptides, most of the Lys-C peptides were present in all samples. Those peptides corresponding to amino acid sequences from 1 to 28 (L1–L2) and 76 to 131 (L7–L9) were identical in all five MUPs analyzed. A further two peptides (L10 and L11) were variably present, due to a known amino acid polymorphism (Lys_{140} to Glu_{140}) that modifies a Lys-C site (Robertson et al., 1997) and, thus, in some proteins, the peptide (L10 + L11 − 18Da) was apparent. All other sequence variability in these proteins was located in peptides L4 and L5. For the Lys-C digest of the 18,682-Da protein (peak 2, expressed by all but one of the May mice), the expected 2095.9-Da L5 peptide was not present in the spectrum. Instead, a smaller 2047.4-Da peptide was present in the spectrum and is likely to correspond to the L5′ peptide arising from a $Phe_{56} \rightarrow Val_{56}$ mutation, as seen in uMUP-VII from BALB/C (Genbank accession X00907) (Kuhn et al., 1984;

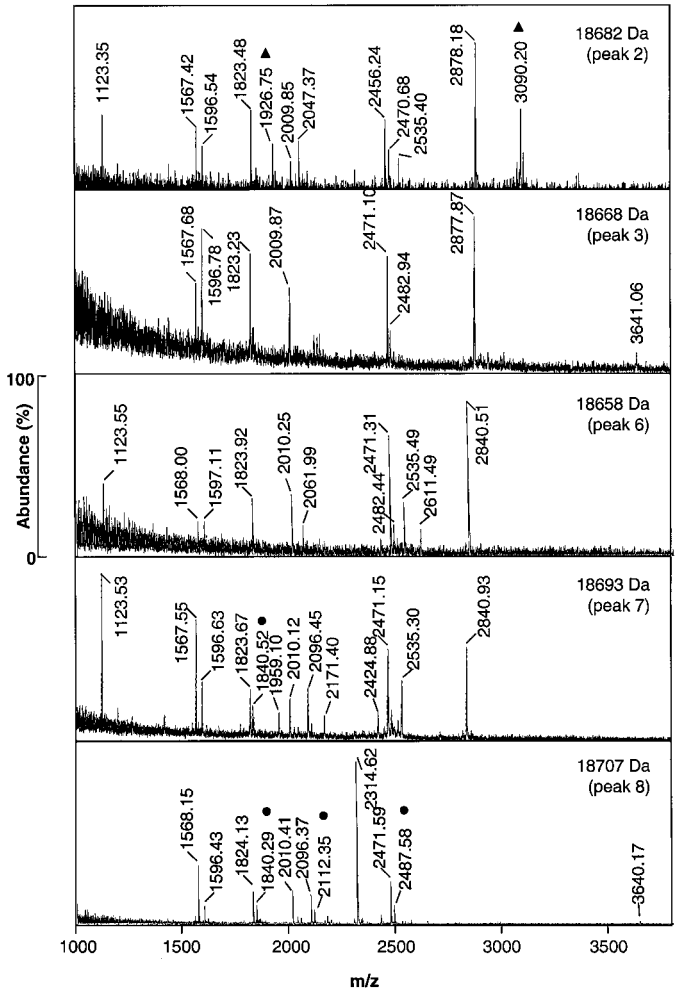


FIG. 5. MALDI-TOF mass spectrometry analysis of Lys-C digested purified M22 urinary proteins. Ion exchange chromatography-purified proteins were denatured in 4 M guanidine thiocyanate and desalted into Lys-C digestion buffer (25 mM Tris, 1 mM EDTA, 2 mM β-mercaptoethanol, pH 8.5) and digested with Lys-C overnight at 37°C. The peptides were analyzed on a PerSeptive Voyager Elite MALDI-TOF MS in reflectron mode. (**A**) Peak 2 (18682 Da), (**B**) Peak 3 (18669 Da), (**C**) Peak 6 (18658 Da), (**D**) Peak 7 (18693 Da), and (**E**) Peak 8 (18707 Da). Peaks marked with a closed circle are +18.003 Da peaks representing the oxidized methionine variant of the parent peak at lower mass. This serves as a useful confirmation of the presence of methionine in these peptides and their assignment to the peptide map. Peaks marked with a triangle are derived from autolysis of endopeptidase Lys-C (L1: 3090.49 Da and L2: 1926.83 Da).

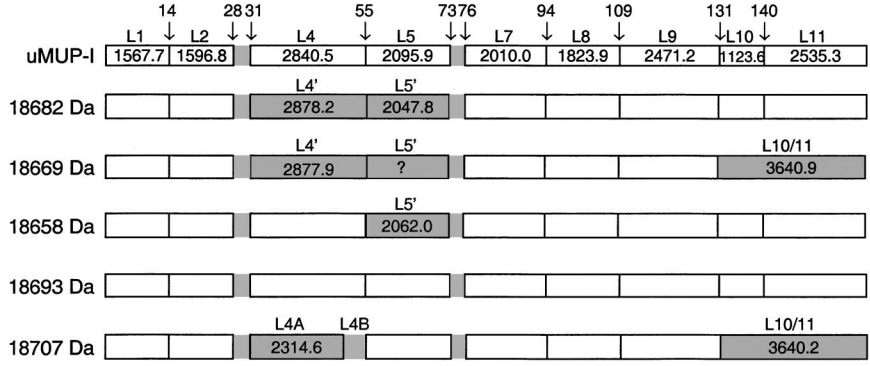


FIG. 6. Comparison of peptides obtained from Lys-C digestion of May mouse urinary proteins with that of a theoretical uMUP-I digest. A theoretical Lys-C digest of the protein sequence for uMUP-I was calculated using MassLynx software (Micromass, Manchester, UK) and compared with the MALDI-TOF results for the five Isle of May mouse peaks peaks. Lys-C peptides are numbered L1–L11. Some peptides are too small to be seen on the MALDI-TOF (L3, L6, and L4B). Blank boxes represent M22 peptides identical to the uMUP-I peptide. Peptide masses corresponding to known mutations found in inbred mice were also seen: $Asn_{50} \rightarrow Lys_{50} = 2314.2$ Da L4A and 559.4 Da L4B peptides, $Phe_{56} \rightarrow Val_{56} = 2047.9$ Da L5′ peptide, and $Lys_{140} \rightarrow Glu_{140} = 3640.8$ Da L10/11 peptide.

Robertson et al., 1997; Marie et al., 2001). Assuming that this was the L5′ peptide, an additional mass change had to account for the difference in the protein mass (10.7 Da smaller that uMUP-I). The 2840.5-Da L4 peptide was not present in the spectrum, but a prominent ion at 2878.2 Da was seen. This is 37.7 Da larger than the uMUP-I L4 peptide, and could, therefore, be a mutated L4′ peptide. The only single mass change of 38Da would be consequential to a Val to His mutation. Peptide L4′ contains two valyl residues, and it is not possible to be specific about which has been mutated. However, a Val to His mutation would also make the protein more cationic and less able to bind to the cation exchange column, which is exactly what is observed (Figure 2). The evidence clearly points to a new allelic variant, and this new protein was termed uMUP-XV according to the suggested nomenclature.

For the Lys-C digests of the 18,668-Da protein (peak 3, expressed by 14/18 males sampled), the L4, L5, L10, and L11 peptides of uMUP-I were not observed. All Lys-C peptide spectra for this protein contained ions at 3641 Da, which corresponds to the L10/11 peptide arising from the deletion of a Lys-C site by the mutation of Lys_{140} to Glu_{140}, as previously seen in the BALB/C protein uMUP-VIII [20]. The 2877.9-Da fragment is likely to be the L4′ peptide that was present in the digest of peak 2 material. Taking all these mass differences into account, peptide L5 would be expected to be 2037 Da. However, no peptide of this mass was present in any spectrum examined. It may be that L5′ in this protein contains

an additional Lys-C cleavage site that renders it too small for analysis. However, the implication is clear, inasmuch as there must be a further amino acid change in L5, which is the only peptide not accounted for. No peptides of the correct mass (i.e., the mass that would account for the remaining protein mass difference) were seen, and no substitution to Lys, thus generating an additional Lys-C cleavage site, could account for the mass differences or any observed peaks in the spectra. This too must be a new protein and is designated uMUP-XVI.

Lys-C digests of the 18,658-Da protein (peak 6, expressed by 10/16 males sampled) had only the L5 peptide mass peak missing, and the difference in protein mass must reside in this peptide. A 2062-Da peak was seen in the MALDI-TOF spectra, 34 Da smaller than the uMUP-I L5 fragment. This protein was termed uMUP-XVII. The third new MUP (uMUP-XVII) differed from uMUP-I at only one peptide: L5, by a decrease in mass of 34 Da. A number of single amino acid substitutions could account for this mass difference, of which a Phe→Ile/Leu substitution was deemed the most likely change. First, this was the only mutation that required a single base change. Second, the position at which the mutation occurred was F56, at which we had previously observed another mutation to valine (Kuhn et al., 1984; Robertson et al., 1997). Finally, the predicted average mass of the protein containing this mutation was 18,559 Da, in close agreement with the observed mass of 18,558 Da. This residue is directed in the central calyx of the MUP structure, and we have demonstrated that the F56V variant demonstrates altered binding of fluorescent probes (Marie et al., 2001). Thus, uMUP-VII from BALB/C, uMUP-X from C57BL/6J, and now uMUP-XVII from Isle of May mice are all variant at this position. All the amino acids at this position are hydrophobic, consistent with their disposition towards the hydrophobic central calyx. UMUP-XVII eluted at the same position on ion exchange chromatography as uMUP-VII from BALB/C, further evidence for the absence of amino substitutions that would evince a charge shift (such as Arg to Glu).

MALDI-TOF mass spectrometry of the Lys-C digest of the 18,693-Da protein (peak 7, expressed in all 17 samples, Figure 3), matched exactly that of uMUP-I. This protein had the same overall charge (i.e., it eluted at the same volume in IEC), the same mass, and resulted in the same Lys-C peptides as uMUP-I, suggesting that it was the same protein. Similar data were obtained for the 18,707-Da protein (peak 8, expressed in all but one of 17 samples, Figure 3), which was identical to uMUP-IX with respect to overall charge, mass, and pattern of Lys-C peptides. These two proteins, initially characterized in inbred mouse strains were, thus, also present in this wild population.

From the five purified proteins mapped by MALDI-TOF mass spectrometry, three new MUPs were identified, each containing at least one mutation. Our previous investigations of MUPs in wild house mice have revealed a much higher degree of heterogeneity than in inbred mice, both as to the number of MUPs expressed per mouse and profile differences between mice even from the same population

(Robertson et al., 1997; Pes et al., 1999; Payne et al., 2001). Although the degree of MUP molecular heterogeneity in Isle of May mice is less than that from other wild mainland populations (Payne et al., 2001), and the extent of sharing of the major protein peaks is substantial, individual MUP profiles were generally more complex than inbred mice, and there was substantially more individual variability in the expression of minor MUP species. This is consistent with selection pressure to maintain a high level of heterozygosity at the MUP loci as predicted.

Although roles for MUPs in ligand release (Hurst et al., 1998) and in direct signaling (Humphries et al., 1999; Brennan et al., 1999) have been established, neither function offers an explanation for the considerable polymorphisms in the MUP family or the combinatorial diversity that ensues from coexpression of multiple proteins in a single urine scent mark. We have proposed that the patterns of MUPs in a scent mark might provide a nonvolatile, stable, and complex label of ownership of the scent mark and, thus, deliver the complex olfactory signal of health, sexual status, and relatedness in the context of an individual animal (Beynon et al., 2001; Malone et al., 2001). We have shown that MUPs contribute to individuality signaling in urine deposits (Hurst et al., 2001) although we do not yet know the relative contribution of the major and minor protein species to this process. This would imply a receptor, or family of receptors, for the MUPs, and the evidence for such a receptor is accumulating. Analysis of the primary sequence of uMUPs, whether from cDNA sequences, where there is direct proof of expression, or of new proteins that we have analyzed by mass spectrometry indicates that much of the heterogeneity resides in endopeptidase LysC limit peptides L4 and L5 (Figure 7).

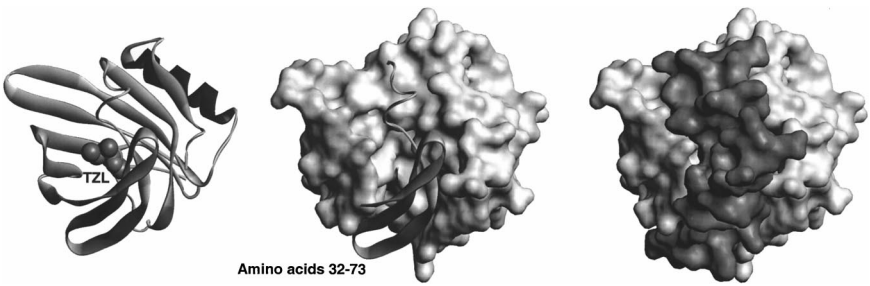


FIG. 7. Three-dimensional structure of MUP showing location of polymorphic variation. The structure of MUP [1MUP.PDB (Bocskei et al., 1992)] was used to generate these views of the protein. The left hand panel is a simple ribbon representation, showing the secondary structure and the approximate position of the ligand 2-*sec*-butyl-4,5-dihydrothiazole [although the disposition of this ligand has recently been revised (Zidek et al., 1999)]. Center panel: the segment of the polypeptide chain representing amino acids 32–73 (Lys C peptides L4 and L5, see text) is displayed in ribbon structure, superimposed on a space filling representation of the remainder of the protein. The right hand panel is a space filling representation in which the L4–L5 segment is shaded darker.

This area of the protein forms an extended patch on the surface of the protein, extended into a beta configuration. In such an extended conformation, the residues both protrude into the central cavity and towards solvent. If there were a selection for amino acid diversity directed to this patch of the surface, it might also lead to changes in cavity residues, which might, therefore, be consequential to the surface effects. Our initial analyses of the kinetics of ligand release have indicated that there are no major differences in the overall release–time curve when a comparison is made of urine or MUP samples derived from different strains and containing different proteins (Robertson et al., 2001). However, such studies do not reflect the environment proximal to a putative MUP receptor, and it is not unreasonable to consider coupling between externally facing residues that interact with a receptor and internally facing cavity-forming residues, such that the binding of the former alters cavity binding characteristics and ligand release.

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HERITABLE VARIATION OF SEX PHEROMONE COMPOSITION AND THE POTENTIAL FOR EVOLUTION OF RESISTANCE TO PHEROMONE-BASED CONTROL OF THE INDIAN MEAL MOTH, *Plodia interpunctella*

GLENN P. SVENSSON,* CAMILLA RYNE, and CHRISTER LÖFSTEDT

Department of Ecology
Lund University
SE-223 62 Lund, Sweden

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Abstract—The short-term evolutionary effect of pheromone-based mating disruption on the mating ability of the Indian meal moth, *Plodia interpunctella*, was investigated. Three independent selection lines were established, and the mating ability of moths in plastic tents treated with high doses of pheromone and in control tents was compared for two consecutive generations. In addition, the heritability of the sex pheromone blend, measured as the ratio of two major pheromone components (*Z*,*E*)-9,12-tetradecadienyl acetate and (*Z*,*E*)-9,12-tetradecadienol, was estimated. Based on a mother–daughter regression analysis including 21 families, the heritability of the pheromone blend was 0.65 ± 0.14, indicating a potential for evolutionary change of the character. However, no increase in mating ability of females in pheromone-treated tents or alteration of the pheromone blend was observed in any selection line when compared with control lines, indicating no or weak selection on the pheromone blend as well as other traits influencing mating ability of this species under the created mating disruption conditions. Factors contributing to the lack of selection effects are discussed.

Key Words—Indian meal moth, *Plodia interpunctella*, Lepidoptera, Pyralidae, sex pheromone blend, mating disruption, heritability, selection.

INTRODUCTION

Mate-signaling in Lepidoptera is often achieved by sex pheromones produced by females to attract conspecific males. Application of synthetic sex pheromones for mating disruption is a successful method for population control of several moth

* To whom correspondence should be addressed. E-mail: glenn.svensson@ekol.lu.se

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species (reviewed by Cardé and Minks, 1995). However, treatment by synthetic pheromones could theoretically result in strong selection leading to evolutionary change of the pheromone communication channel, which could reduce the efficiency of the control method. The potential for development of resistance towards pheromone-based mating disruption would depend on the heritable variation of traits involved in pheromone production and perception. Although sex pheromone systems in moths are often strongly canalized, substantial variation in pheromone-related characters has been revealed in several species (reviewed by Löfstedt, 1990).

Phenotypic variation in pheromone communication systems within and between populations is rarely taken into account when using pheromone-based control strategies, i.e., only the major pheromone component(s) or the average multicomponent blend are used as disruptants in field applications. It can be hypothesized that in species showing large phenotypic variation in pheromone-associated traits, females producing high amounts of pheromone may have a higher chance to attract searching males under mating disruption conditions than females producing low amounts of pheromone. Similarly, females producing skewed ratios of pheromone components compared to the applied synthetic background may be recognized more easily by the males. Evidence for increased emission rates in females after long-term exposure to high doses of pheromone has been reported from both field and laboratory studies (Haynes and Baker, 1988; Shani and Clearwater, 2001). So far, no correlated effect on pheromone blend production has been observed (Haynes and Baker, 1988; Shani and Clearwater, 2001). However, many studies have shown that single pheromone components disrupt mating less efficiently compared to more complex blends (Minks and Cardé, 1988), indicating that additional components produced by females may be exploited by males in a pheromone-permeated atmosphere, and that there is a potential for selection for altered blends under mating disruption conditions.

The heritability (h^2) of a character is measured as the fraction of the total phenotypic variance that is attributable to additive genetic factors (Falconer and Mackay, 1996), i.e., how much of the variance observed is subject to selection. Thus, estimates of heritability can be used to predict responses to selection and the potential for evolutionary change of characters. The genetic basis for pheromone production and perception has been examined in several moth species (e.g., Roelofs et al., 1987; Hunt et al., 1990; Foster et al., 1996; LaForest et al., 1997). Significant heritability has been reported for both female pheromone blend composition (Collins and Cardé, 1989) and titer (Collins et al., 1990), as well as male response to sex pheromones in the pink bollworm moth, *Pectinophora gossypiella* (Collins and Cardé, 1990). Heritability studies have also been performed on the communication system of the red-banded leafroller moth, *Argyrotaenia velutinana* (Roelofs et al., 1986; Sreng et al., 1989), the black cutworm moth, *Agrotis ipsilon* (Gemeno et al., 2000), and the cabbage looper moth, *Trichoplusia ni* (Gemeno et al., 2001).

Although selection studies have been performed on pheromone communication traits in several moth species (e.g., Collins and Cardé, 1989; Sreng et al., 1989; Collins et al., 1990; Liu and Haynes, 1994; Shani and Clearwater, 2001), in most cases only one selection line has been analyzed, which makes it impossible to distinguish true selection effects from effects caused by genetic drift. To be able to separate these mechanisms, several independent selection lines have to be analyzed.

The Indian meal moth, *Plodia interpunctella*, is a serious indoor pest on stored products. The first pheromone component identified in this species was (*Z*,*E*)-9,12-tetradecadienyl acetate (*Z*9,*E*12–14:OAc) (Kuwahara et al., 1971). Later, Kuwahara and Casida (1973) and Sower et al. (1974) identified (*Z*,*E*)-9,12-tetradecadienol (*Z*9,*E*12–14:OH) as a second pheromone component, and Vick et al. (1981) showed that by adding the alcohol to the acetate, trap catch of males increased significantly. Recently, Teal et al. (1995) and Zhu et al. (1999) identified (*Z*,*E*)-9,12-tetradecadienal (*Z*9,*E*12–14:Ald) and (*Z*)-9-tetradecadienyl acetate (Z9–14:OAc) as additional pheromone components of the species. Phenotypic variation in both pheromone titer and ratios of individual components within the pheromone blend has been observed in this species (Zhu et al., 1999). Small-scale mating disruption experiments have recently been performed on *P. interpunctella* (Ryne et al., 2001). Mating was suppressed by up to 93% using different doses and composition of pheromones. However, mating disruption was not complete in any of the trials, indicating that some females may be easier to find by males against a background of synthetic pheromone.

In the present study, we investigated: (1) if the variation observed in pheromone blends produced by female *P. interpunctella* is heritable, and (2) if there is a potential for rapid evolution of resistance to synthetic pheromones due to a shift in the pheromone blend.

METHODS AND MATERIALS

Insects. Males and females of *P. interpunctella* were obtained from a laboratory culture maintained at the Department of Ecology at Lund University for 35 generations. This culture was established from a laboratory culture at the Danish Pest Infestation Laboratory at Lyngby, Denmark. About 100 males and 100 females from the Central Science Laboratory, Slough, England, were incorporated into the culture after ca. 20 generations. Larvae were reared on an artificial diet described by Zhu et al. (1999) at 24°C and 60% relative humidity. Pupae were separated according to sex and placed in a 17L:7D photoperiod at 22°C and 60% relative humidity.

Chemicals and Dispensers. The synthetic compounds *Z*9,*E*12–14:OAc, *Z*9,*E*12–14:OH and *Z*9–14:OAc were all purchased from Bedoukian Research Inc., Danbury, Connecticut, USA, and *Z*9,*E*12–14:Ald was synthesized from *Z*9,*E*12–14:OH at the Department of Chemistry, Royal Institute of Technology, Stockholm, Sweden. A blend containing *Z*9,*E*12–14:OAc, *Z*9,*E*12–14:Ald,

*Z*9,*E*12–14:OH, and *Z*9–14:OAc, in the ratio 100:11:18:12 (Zhu et al., 1999), was used as disruptant. A controlled-release system (MSTRS) described by Mafra-Neto and Baker (1996) was used for emission of pheromone.

Gland Extract Analysis. Pheromone gland extracts of individual female *P. interpunctella* (2–4 days old) were dissected 1–3 hr into the scotophase and extracted in 5 μl of hexane with internal standard (2.5 ng/μl of *Z*8–13:OAc). Extracts were analyzed on a Hewlett Packard (HP) 5890 Series II gas chromatograph equipped with a flame ionization detector (FID) and a HP-INNOwax capillary column (30 m $\times$ 0.25 mm ID, 0.25-μm film thickness). Hydrogen was used as carrier gas. Samples were injected in the splitless mode, and the injector temperature was 225°C. The column temperature was maintained at 80°C for 2 min after injection and then linearly increased to 230°C at a rate of 10°C/min.

Heritability Estimate. To test the potential for evolutionary change of the sex pheromone blend, the heritability of the ratio of two major components, *Z*9,*E*12–14:OAc and *Z*9,*E*12–14:OH, was estimated. The two additional components in the pheromone blend, *Z*9,*E*12–14:Ald and *Z*9–14:OAc, were not included in the analysis, as these were not reliably quantified in all females. Sex pheromone production of female *P. interpunctella* stops after mating. However, after oviposition, which normally occurs two to three days after mating, about 50% of the females initiate calling again and may attract males for remating (personal observations). The repeatability of the pheromone blend in females has been shown to be high in the different moth species investigated so far (Barrer et al., 1987; Du et al., 1987; Witzgall and Frérot, 1989; Svensson et al., 1997). Assuming that this is also the case for *P. interpunctella*, a female should produce the same pheromone blend after oviposition as before mating.

Pairs of 1- to 2-day-old males and females were placed in 250-ml plastic jars with artificial diet for mating. After oviposition, pheromone glands were dissected from calling females and analyzed as described above. Larvae originating from matings were kept in jars with artificial diet. Component ratios were analyzed in three or four daughters per family. Mean component ratios of daughters were then plotted against their corresponding mothers and heritability was estimated as twice the slope of the standardized mother–daughter regression line according to Falconer and Mackay (1996).

Selection Effects in Pheromone-Permeated Air. Experiments were performed in transparent plastic tents (2.5 $\times$ 2.5 $\times$ 2.5 m) in a greenhouse at Lund University, Sweden. The tents were purchased from the Central Science Laboratory, Slough, England. Control and treatment tents ($N = 2$) were placed in separate chambers to avoid chemical interference. Conditions during the experiments were: 22–28°C and 28–44% relative humidity. Passive ventilation was provided by creating two net-covered holes (10 $\times$ 15 cm) on opposite walls of each tent. In treatment tents, a MSTRS device was placed at 1 m height and programed to release pheromone every 15 min 24 hr/day. No MSTRS were used in control tents. The four-component

disruptant was diluted in 95% ethanol to correspond to a release rate of 50 μg/min of *Z*9,*E*12–14:OAc. The studies by Mafra-Neto and Baker (1996) and Ryne et al. (2001) were used as guidelines to choose the concentrations released from MSTRS devices. Those studies resulted in suppression of mating by up to 93% and 100% for *P. interpunctella* and almond moths, *Ephestia cautella*, respectively (Mafra-Neto and Baker, 1996; Ryne et al., 2001). Before each experimental period, pheromone was emitted from MSTRS for 48 hr to build up sufficient background concentrations of the disruptant. Between experimental periods, treatment tents were ventilated.

Virgin moths were released into control tents and pheromone-treated tents (10 males and 10 females per tent) and recaptured individually after 24 hr. The density used should be relevant to those under natural indoor conditions, as this species often aggregates. Females from control tents were dissected and checked for the presence of spermatophores, indicating successful mating. Females from treatment tents were put individually into plastic jars with artificial diet for oviposition. After two weeks, the presence of larvae in jars was scored and the mating frequency was estimated. The mating ability of moths from these selection families, as well as unselected moths randomly sampled from the laboratory culture, was then estimated in control tents and treatment tents for two consecutive generations. Only females from selection families in treatment tents were placed in jars for oviposition, i.e., females from selection families in control tents as well as females from the laboratory culture in control and treatment tents were dissected and checked for spermatophores. Up to 16 families originating from mated females in treatment tents were used in the next generation. In total, three independent selection lines were created and run in the same way as described above.

Differences in mating frequencies between moths in pheromone-treated tents and control tents in the initial generation of each selection experiment were analyzed by unpaired *t* tests. In consecutive generations, one-way ANOVAs followed by Tukey's test were applied both within and between lines. Mating frequencies were arcsin $\sqrt{x}$-transformed before analysis. In addition, virgin female offspring from five families in the second generation of each selection line as well as virgin females from the laboratory culture were randomly sampled and the ratio of *Z*9,*E*12–14:OAc and *Z*9,*E*12–14:OH was analyzed. Differences in component ratios between selection lines and unselected moths from the laboratory culture were compared using unpaired *t* test on arcsin $\sqrt{x}$-transformed values.

RESULTS

Heritability Estimate. In total, 21 families were analyzed for pheromone component ratios. Based on a mother–daughter regression, heritability ($\pm$SE) of the sex pheromone blend was estimated to be 0.65 ± 0.14 (Figure 1), calculated as twice

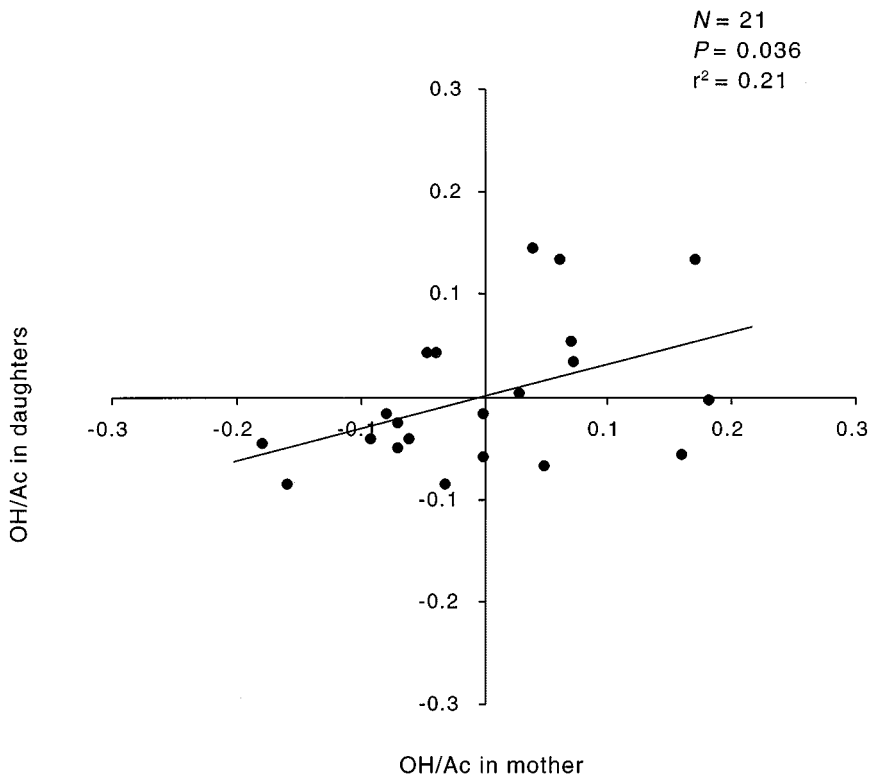


FIG. 1. Mother–daughter regression on ratios of *Z*9,*E*12–14:OH and *Z*9,*E*12–14:OAc based on pheromone gland extracts from 21 families of *P. interpunctella*. Axes intersect at the mean values of all mothers and all daughters. Heritability equals twice the slope (0.325) of the regression line.

the slope of the regression line (slope significantly different from zero, $P = 0.036$). The curve fit was relatively poor ($r^2 = 0.21$). Fifty-four percent (21 of 39) of the mated females initiated calling after oviposition and could be used for gland analysis. Pheromone was absent or found in very small amounts in glands of mated females not performing calling behavior after oviposition. A paired *t* test on ratios of the two pheromone components used in the heritability estimation revealed no difference between mated mothers and the corresponding virgin daughters (0.30 ± 0.02 vs 0.31 ± 0.02, $T = 0.28$, $P > 0.05$). This indicates that the heritability estimate based on parent-offspring regression was not affected by altered pheromone blends produced by mated mothers. In contrast, both pheromone components were produced in smaller amounts in mated mothers compared to their virgin daughters (*Z*9,*E*12–14:OAc: 0.71 ± 0.13 ng vs. 1.62 ± 0.23 ng, $T = 3.47$,

$P < 0.01$; $Z9,E12$–14:OH: 0.21 ± 0.04 ng vs. 0.51 ± 0.08 ng, $T = 3.49$, $P < 0.01$). Therefore, no heritability estimates of quantities of individual components could be performed using parent–offspring regressions.

Selection Effects in Pheromone-Permeated Air. Mating frequencies of moths in pheromone-treated tents and control tents in different generations from three independent selection lines as well as unselected moths are shown in Figure 2. Significant mating disruption in treatment tents compared to control tents was achieved in all experiments. However, the mating frequency in pheromone-treated tents did not differ between selection lines and unselected moths within any generation as would be expected if selection for increased mating ability in pheromone-permeated air had occurred. Mating frequencies of both selection lines and unselected moths remained at a high level in untreated tents throughout the experiments (Figure 2). However, statistical differences in mating frequency were found between selection lines (Figure 2). Thus, data could not be pooled. No difference in ratios of $Z9,E12$–14:OAc and $Z9,E12$–14:OH could be detected between unselected females and selection line females (Figure 3).

DISCUSSION

The present study focused on the short-term evolutionary effect of pheromone-based mating disruption on the mating ability of *P. interpunctella*. By combining heritability and selection studies, we tried to evaluate the potential for resistance development towards mating disruption strategies via evolutionary change of the pheromone blend. The heritability of the ratio of the two major components was estimated to be 0.65, indicating that a significant proportion of the phenotypic variation was due to additive genetic factors and that there should be potential for an evolutionary change of the character. Significant heritability of pheromone component ratios has been reported for other moth species (Table 1). Although the pheromone blend showed high heritability, no significant shift in component ratio

TABLE 1. ESTIMATED HERITABILIY OF TRAITS INVOLVED IN PHEROMONE COMMUNICATION IN MOTHS

Species	Parameter	h^2	Reference
Agotis ipsilon	Pheromone titer	0.56–1.15	Gemeno et al. (2000)
	Blend ratio	0.56–1.09	Gemeno et al. (2000)
Argyrotaenia velutinana	Blend ratio	0.41–0.64	Sreng et al. (1989)
Pectinophora gossypiella	Pheromone titer	0.71	Collins et al. (1990)
	Blend ratio	0.50	Collins and Cardé (1989)
	Male response	0.16	Collins and Cardé (1990)
Plodia interpunctella	Blend ratio	0.65	Present study
Trichoplusia ni	Pheromone titer	0.20–1.13	Gemeno et al. (2001)

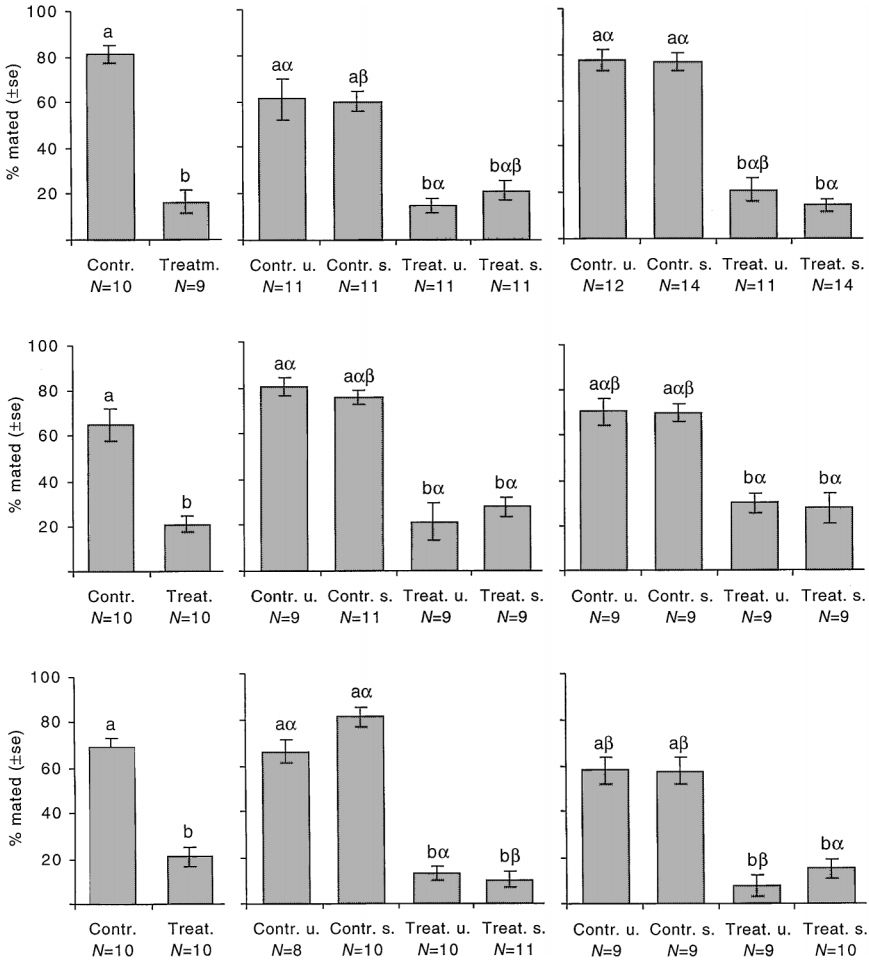


FIG. 2. Effect of selection on mating ability of *P. interpunctella*. Mating frequencies (mean ± SE) of moths from three separate selection lines (s) and of unselected moths from the laboratory culture (u) estimated in control tents or in treatment tents with disruptive doses of sex pheromone for three generations. Bars with different letters are significantly different (one-way ANOVA followed by Tukeys test, $P < 0.05$). English letters show significant differences between groups within each generation of the same line. Greek letters show significant differences within the same generation of one group, e.g., unselected moths in control tents, between lines.

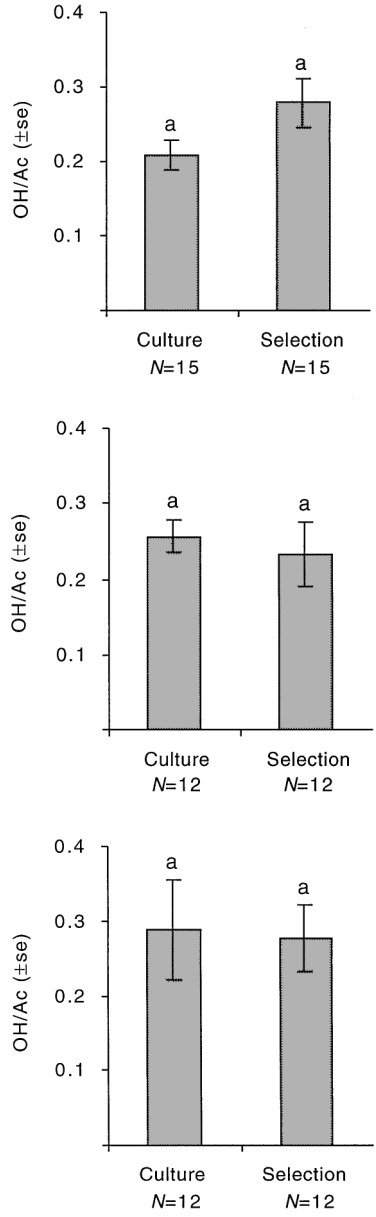


FIG. 3. Ratios (mean ± SE) of *Z*9,*E*12–14:OH and *Z*9,*E*12–14:OAc analyzed from pheromone gland extracts of *P. interpunctella*. Female offspring from the second generation of selection from three independent selection lines were analyzed as well as unselected females from the laboratory culture. Component ratios did not differ between groups in any experiment (unpaired t test, $P > 0.05$).

could be observed in third-generation females from three independent selection lines when compared with unselected females from the laboratory culture. Furthermore, no increase in mating ability under mating disruption conditions was observed in any selection line when compared to control moths. Since mating frequencies of selection lines in control tents were as high as for unselected moths, the lack of selection effect could not be attributable to overall lowering of mating due to inbreeding. Instead, our data indicate weak or no selection on the pheromone blend as well as other traits influencing mating ability of this species under the mating disruption conditions created in this study. The coefficient of the parent–offspring regression curve was relatively low, showing that the curve explained only a small fraction (21%) of the variation found in pheromone component ratios. As the curve fit was poor, the heritability may have been overestimated. The relatively small sample size and maternal effects could be factors explaining the low r^2 value.

Few studies have investigated how selection may affect the potential for resistance development towards synthetic pheromones in moths. Torres-Vila et al. (1997) performed a selection experiment on the European grape vine moth, *Lobesia botrana*. Mating in olfactometers permeated with high doses of the major sex pheromone component, (*E*,*Z*)-7,9-dodecadienyl acetate, significantly increased after only one generation of selection. However, the correlation between mating ability and pheromone production was not evaluated in that study. Furthermore, Torres-Vila et al. (1997) used only the major component of the sex pheromone as disruptant, whereas a multicomponent blend was used for disruption in the present study, which may have limited the potential for resistance development. Shani and Clearwater (2001) analyzed the effect on pheromone production in *E. cautella* females exposed to high doses of pheromone. Although females from the selection population produced significantly more pheromone compared to the nondisrupted population after seven generations of selection, overall pheromone production declined drastically in the disrupted population as well as the nondisrupted population during the selection experiment. However, the effect on pheromone production was not associated with an increased mating ability of the disrupted population in pheromone-permeated air when compared with the nondisrupted population (Shani and Clearwater, 2001). Haynes and Baker (1988) showed that *P. gossypiella* females from a population exposed to high doses of synthetic pheromones emitted significantly more pheromone during 1985 compared to 1982 and 1983. Although the increase in pheromone emission observed in these two studies was minor and would probably not result in effective resistance to mating disruption, it could be a result of this new selection pressure imposed on the populations.

In the majority of cases where selection effects on moth pheromone communication systems have been shown (e.g., Collins and Cardé, 1989; Sreng et al., 1989; Collins et al., 1990; Shani and Clearwater, 2001), only one selection line has been used, which makes it impossible to separate true selection effects from

effects caused by genetic drift. This is clearly shown in the study by Sreng et al. (1989) where a two-way selection scheme was carried out to change the ratio between two pheromone components of *A. velutinana*. One "high-ratio" line and one "low-ratio" line were run for three generations of selection and then maintained by random mating for six additional generations. Whereas no change in composition of the pheromone blend occurred in the low-ratio line after the selection pressure was removed, the blend in the high-ratio line continued to change in the same direction and at almost the same rate as during the intense selection. Genetic drift is the most probable cause for this asymmetry in change between the two lines.

The only clear evidence so far of a change in a pheromone communication system via selection is in the cabbage looper moth, *Trichoplusia ni* (Liu and Haynes, 1994). A mutation occurred in a laboratory culture of this species, causing a dramatic change in the composition of the pheromone produced by mutant females compared to the wild type (Haynes and Hunt, 1990). The mutant colony was split into two colonies and reared separately. Initially, the mutant blend attracted low numbers of conspecific males (Haynes and Hunt, 1990), but after 49 generations of rearing, the male response towards the altered pheromone increased to the same level as to the normal blend. As this change occurred in both colonies, it should reflect a true selection effect and not an effect caused by genetic drift. Recently, a selection experiment was performed on the mating ability of mutant and wild-type *T. ni* in the field under mating disruption conditions using the normal pheromone blend as disruptant (Evenden and Haynes, 2001). Interestingly, for each generation of selection, the relative proportion of mutant females increased in treatment cages compared to control cages. After four generations, the proportion of the mutant phenotype was significantly greater in treated cages compared to control cages. Thus, mating disruption programs using the average pheromone blend may select for females producing skewed blends and this would increase the potential for evolution of resistance to mating disruption.

The fact that we analyzed three independent selection lines without finding any effects on pheromone composition or mating ability suggests that there was at least a limited potential for resistance development to mating disruption in a few generations' time. However, the possibility that resistance towards mating disruption can evolve after more generations of selection cannot be ruled out. As shown by Haynes and Hunt (1990), it may take substantial numbers of generations before any selection effects are observed. Thus, our results are difficult to interpret.

The potential for development of resistance towards mating disruption via selection for altered blends or higher pheromone production in females may be small in natural populations, as pheromone communication systems in moths are often strongly canalized, i.e., genetic correlations among individual components can limit rapid changes of the pheromone blend in response to selection. Genetic correlations of components involved in the pheromone blends of normal and mutant *T. ni* have been investigated by Gemeno et al. (2001). As the strains differ in the

pattern of genetic correlations, they will evolve differentially even when the same selection pressure is imposed. Although large variation in component ratios was found in *P. interpunctella*, the pheromone components were highly correlated to each other ($r^2 \approx 0.90$), indicating a limited potential to change the blend by selection.

Attempts to change the pheromone blend by selection have been performed on *P. gossypiella* (Collins and Cardé, 1989) and *A. velutinana* (Roelofs et al., 1986), two species which utilize geometric isomers as main pheromone components. Statistically significant changes of isomeric ratios were obtained by selection in both species, but they were small. Although large heritable variation in pheromone component ratios was found among females in our laboratory culture of *P. interpunctella*, the results do not support the hypothesis of resistance development towards mating disruption via selection for altered blends. In addition, a broad response window in male moths to pheromone blends may further decrease the probability of such a scenario. However, no data are available on male response to different ratios of the investigated pheromone components of *P. interpunctella*.

A more probable route to resistance may be via point mutations with major effects on the communication channel. Although the chance of finding and evaluating the effect of such rare mutations is strongly limited in laboratory as well as natural populations, the studies on wild-type and mutant colonies of *T. ni* (Haynes and Hunt, 1990; Liu and Haynes, 1994; Evenden and Haynes, 2001) show that changes of the communication channel can occur and may lead to evolution of resistance to mating disruption.

The doses used in our experiments may have been too high to mimic large-scale mating disruption conditions, making it hard to extrapolate our data to future control programs. However, males could probably use other cues than sex pheromone to locate mates in pheromone-permeated air. McNeil (1992) proposed that if there is heritable variation in the ability to exploit other cues than those associated with long-range sex pheromones, selection could increase the chance of locating a mate under mating disruption conditions. *Plodia interpunctella* has a complex mating system involving long-range male attraction to sex pheromones emitted by females as well as short-range communication by olfactory and acoustic cues (McLaughlin, 1982; Trematerra and Pavan, 1995). The relatively high population density of moths in our small-size tent experiments should facilitate short-range communication. However, this was not reflected in any selection effect on mating ability. In addition, insects were removed from the tents after 24 hr, which may have resulted in a limited selection effect compared to if they would have been removed after several days.

To summarize, in spite of significant heritable variation in pheromone production of *P. interpunctella*, we found no evidence for resistance evolution towards mating disruption due to a shift in the pheromone blend. However, due to the few generations of selection conducted in this study, we cannot rule out the possibility

that resistance can evolve after more generations of selection. Long-term selection studies in pheromone-permeated air using independent lines are needed to get a better picture of how populations are affected by high doses of synthetic pheromone to facilitate the development of safe strategies of pheromone-based control of moths.

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COMPARATIVE STUDIES OF FEMALE SEX PHEROMONE COMPONENTS AND MALE RESPONSE OF THE CORN STALK BORER *Sesamia nonagrioides* IN THREE DIFFERENT POPULATIONS

F. D. KROKOS,[1] A. AMELINE,[2] J. BAU,[3] A. SANS,[4] M. KONSTANTOPOULOU,[1] B. FRÉROT,[2] A. GUERRERO,[3] M. EIZAGUIRRE,[4] C. MALOSSE,[2] O. ETCHEPARE,[5] R. ALBAJES,[4] and B. E. MAZOMENOS[1,*]

[1]*Chemical Ecology and Natural Products Laboratory*
Institute of Biology NCSR "Demokritos"
P.O. Box 60228
153 10 Aghia Paraskevi Attikis, Greece

[2]*I.N.R.A., Unité de Phytopharmacie et des Médiateuers Chimiques*
Route de St Cyr
78026 Versailles Cedex, France

[3]*Department of Biological Organic Chemistry, IIQAB (CSIC)*
Jordi Girona 18-26
08034 Barcelona, Spain

[4]*Universitat de Lleida, Centre UdL-IRTA*
Rovira Roure 177
25198 Lleida, Spain

[5]*Fédération de Protection des Cultures*
11000 Carcassonne, France

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Abstract—Studies to determine possible differences in the pheromone communication system of three different populations of the corn stalk borer *Sesamia nonagrioides* (Lef.) in France, Spain, and Greece were carried out. The two main pheromone components (*Z*)-11-hexadecenyl acetate (*Z*11–16:Ac) and (*Z*)-11-hexadecenol (*Z*11–16:OH), were detected in all analyses with very small differences in the three populations. Among the minor components, analyzed by GC-MS on concentrated gland extracts from French and Greek origin females, (*Z*)-11-hexadecenal (*Z*11–16:Ald) was detected in minor amounts. Wind-tunnel and field studies revealed similar a male response in the three populations to pheromone glands extracts and synthetic pheromone regardless of the

* To whom correspondence should be addressed. E-mail: bmazom@mail.demokritos.gr

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female/male origin. The results do not support the assumption of the existence of different pheromone types of the corn stalk borer *S. nonagrioides* due to geographic isolation.

Key Words—*Sesamia nonagrioides*, Lepidoptera, Noctuidae, sex pheromones, (*Z*)-11-hexadecenyl acetate, (*Z*)-11-hexadecenol, (*Z*)-11-hexadecenal, dodecyl acetate, geographic variation.

INTRODUCTION

The corn stalk borer *Sesamia nonagrioides* (Lef.) (Lepidoptera: Noctuidae) is considered the most serious corn pest in many countries distributed across southern Europe between the 45°N parallel and northwest Africa. The insect completes a variable number of generations annually, from two in southern France to up to four in Morocco (Anglade, 1972).

The sex pheromone of *S. nonagrioides* was identified by Sreng et al. (1985) as a mixture of (*Z*)-11-hexadecenyl acetate (*Z*11–16:Ac) (**1**) and (*Z*)-11-hexadecenol (*Z*11–16:OH) (**2**). The two compounds tested separately or as a 90:10 blend were attractive to males in laboratory bioassays, but traps baited with the two-component blend were not effective. The same authors reported that hexadecyl acetate (16:Ac) was present in the pheromone gland extract, but no biological activity was established. Later, the isolation of two additional components, (*Z*)-11-hexadecenal (*Z*11–16:Ald) (**3**) and dodecyl acetate (12:Ac) (**4**), was reported (Mazomenos, 1989). The four-component blend **1** + **2** + **3** + **4** in a 69:8:8:15 ratio increased male attractiveness to the pheromone traps.

Field trials conducted in France and Spain with pheromone traps to monitor moth populations indicated that traps baited with the four-component blend **1–4** in 69:8:8:15 ratio were less attractive than traps baited with virgin females. In addition, high numbers of other sympatric moth species were also captived by these traps (Perdiguer et al., 1992; Frérot et al., 1997a).

An improved formulation for male attractiveness and selectivity was developed by Sans et al. (1997) as a mixture **1–4** in a 77:8:10:5 ratio, based on wind-tunnel and field tests in Spain. However, the new formulation was again less effective than virgin females in field tests carried out in southern France (Frérot et al., 1997a). In this context, genetic differences between Spanish and French populations from certain geographic areas have been shown, and insect movements usually occur through the Atlantic or Mediterranean coast but not across the Pyrenees (Bues et al., 1996). In several noctuid species, differences in the pheromone composition produced by the females and the male responses to pheromone blends of different geographic populations have been well documented (Cardé and Baker, 1984; Phelan, 1993).

We report here comparative studies aimed at determining: (1) possible differences in the sex pheromone communication system of three *S. nonagrioides*

populations in France, Spain, and Greece; (2) male response of the three populations both to natural and synthetic pheromone in wind tunnel and field tests; and (3) cross-mating behavior among the three populations.

METHODS AND MATERIALS

Insects. Moths used in this study were derived from artificially reared colonies maintained in each laboratory. The composition of the artificial diet used in the French and Spanish laboratories has been described by Poitout and Bues (1970). In Greece, the larvae were reared on a diet described by Tsitsipis et al. (1983). Larvae and pupae were maintained at $27 \pm 1^{\circ}$C with a 16L:8D regime and relative humidity of $60 \pm 5\%$. Moths were separated by sex upon emergence and kept in $30 \times 30 \times 30$-cm screen cages in separated chambers until use. Insects at the pupal stage were exchanged among the collaborating groups.

Behavior Studies. Wind-tunnel bioassays were conducted in the three laboratories. The type of wind tunnel and conditions used were $150 \times 35 \times 35$-cm glass tunnel at 25–27°C and 65% relative humidity in Greece; $140 \times 70 \times 40$-cm Plexiglas tunnel at 25–27°C and 80% relative humidity in France, and $180 \times 55 \times 50$-cm glass tunnel at 24–26°C and 65% relative humidity in Spain. In all cases, air from the pheromone-free room was generated by a centrifugal fan and filtered to provide laminar airflow. The air speed through the tunnels was 50 cm/sec. The air inside the tunnels was vented outside via an exhaust tube at the downwind end of the tunnels. Red light (2.5–3 lux) that allowed observation and recording with CCD cameras illuminated the tunnels. Males, 2–3 days old and placed individually in screen cages, were transferred to the test room before the onset of the dark period. Wind-tunnel tests were run 5 hr after lights off when the peak of sexual activity occurred (Babilis and Mazomenos, 1992). The following sequences of behavior were recorded: wing fanning and taking flight, close approach (ca. 10 cm), and contact with the source. Only those males arresting at the pheromone source for a period of 5 sec or more were recorded as contact with the source. The pheromone baits were hung 15 cm apart. Males were introduced in the tunnel 1.2–1.6 m downwind of the pheromone bait, and each test lasted 5 min. The behavior responses of males from the three populations tested were compared using χ^2 homogenity test ($P \leq 0.05$).

Pheromone Sources. Pheromone glands and gland extracts obtained from calling females, as well as the synthetic pheromone adsorbed on a filter paper (Whatman No. 1; 1.5×1.5 cm), were used as baits for the wind-tunnel tests.

Synthetic Chemicals. Synthetic pheromone components were obtained from the Sociedad Española de Desarrollos Químicos, S.A (SEDQ) (Barcelona, Spain). The components were found to be 96–98% pure by GC analysis.

Gland Extraction. The pheromone was collected from the pheromone gland of calling virgin females during the dark period, 5 hr after lights off. Each time, five glands were removed with forceps and extracted in 200 μl of solvent for 30 min. The solvents used were pentane, hexane, and methylene chloride. The extracts were concentrated under a gentle stream of nitrogen and used for behavior studies or analyzed by GC-MS. If they were not immediately used, the extracts were stored at -20°C.

Solid-Phase Microextraction (SPME). For the SPME technique, the fiber used for pheromone collection was a 7-μm polydimethyl siloxane (Supelco Inc., Bellefonte, Pennsylvania, USA). The fiber was gently rubbed on the surface of the gland of a calling female for few minutes, and the chemicals adsorbed on the fiber were directly analyzed by GC or GC-MS (Frérot et al., 1997b).

GC and GC-MS Analyses. GC and GC-MS analyses of gland extracts from the three colonies were carried out in each laboratory. Particular attention was paid not only to the detection of major pheromone components of the extracts but also to the presence of other possible minor components that might contribute to increase male attraction. Electron impact ionization (EI) mass spectra (70 eV) were obtained under SCAN or SIM mode. The following instruments and conditions were used: In Greece, an HP-5890 Series II gas chromatograph interfaced to a Fisons VG Trio 1000 mass spectrometer was used with a DB-5 (J&W Scientific) fused silica capillary column 60 m $\times$ 0.25 mm ID, programmed at 50°C for 2 min, to 250°C at 5°C/min for 1 min, and then to 280°C at 2°C/min. In France, a Delsi-300 gas chromatograph interfaced with a Nermag R30-10 mass spectrometer and with a DB-5 (J&W Scientific) capillary column 25 m $\times$ 0.32 mm ID was used. The GC conditions were injection at 120°C and programmed to 260°C at 10°C/min. In Spain, a Fisons gas chromatograph coupled to a Fisons MD-800 mass selective detector with a HP-1 (Hewlett Packard) capillary column 30 m $\times$ 0.20 mm ID was utilized. The GC conditions were injection at 100°C and programmed to 250°C at 5°C/min, and then to 280°C at 10°C/min. Compounds in the gland extracts and SPME fiber were identified by comparison of the retention times and mass spectra with those of authentic standards.

Field Tests. Field trials were conducted in Catalonia (northeast Spain), Aude (southern France), and Kopais (central Greece). The experimental design, type of traps (Universal Moth Traps), and pheromone dispensers used were the same for the three countries. Rubber septa (Aldrich Ltd, No 10070-6), used as dispensers, were loaded with 100 μg of the two pheromone blends of $\mathbf{1}+\mathbf{2}+\mathbf{3}+\mathbf{4}$ in 69:8:8:15 and 77:8:10:5 ratios. Dispensers were prepared in Greece and mailed to the other groups. Funnel traps were installed at 1.5 m height, 1 m from the corn margin at the four corners of each field. Baited traps were in operation from April until late October, the traps were rotated clockwise weekly, and males captured were recorded and removed. Pheromone dispensers were renewed every two months.

Statistics. For statistical analysis, trap captures within sites were analyzed by using the paired t test ($P \leq 0.05$). The day effect was eliminated by averaging the trap captures for each of the two blends tested. Prior to analysis, the data were $[\log_{10}(x + 1)]$ transformed.

RESULTS

Male Response to Pheromone Glands and Gland Extracts from Calling Females. These studies were carried out to establish whether females attract nonsympatric males. A series of tests were done with excised pheromone glands from calling French females as the attractant source for Spanish, Greek, and French males. Almost all males tested (86–100%) initiated flight, and among them 52–72% successfully contacted the source (Table 1). Among the three populations, Spanish males displayed a higher response in all behavioral steps than the other two strains, but no significant differences were observed.

The effect of gland extracts from calling females on males' response was also studied in two independent series of experiments when extracts of 1 and 4 female equivalents (FE) were used as baits. Males of the three populations were similarly attracted (Table 1). The proportion of males that displayed close approach or made contact with the pheromone source was higher in 4 FE bait compared to the 1 FE bait. No difference was detected at the same concentration; i.e., males from different geographical origins showed the same responses.

TABLE 1. PERCENTAGE OF *Sesamia nonagrioides* MALES RESPONDING IN WIND TUNNEL TO FEMALE PHEROMONE GLANDS AND PHEROMONE GLAND EXTRACTS[a]

Pheromone source	Males origin	Behavior steps[b]		
		Take off	Close approach	Landing
Experiment 1 (pheromone gland)				
French	French	86[a]	68[b]	52[c]
French	Greek	95[a]	72[b]	64[c]
French	Spanish	100[a]	76[b]	62[c]
Experiment 2 (gland extract, 1 FE)				
French	Greek	95[a]	54[b]	27[c]
Greek	Greek	96[a]	58[b]	31[c]
Experiment 3 (gland extract, 4 FE)				
Spanish	Spanish	77[a]	77[b]	77[c]
Spanish	Greek	91[a]	76[b]	65[c]

[a] Glands and extracts were obtained from calling females of the three populations ($N = 20$, experiment 1; $N = 15$, experiment 2; $N = 30$, experiment 3).

[b] For each experiment, values within the same type of behavior and with the same letter are not significantly different (χ^2 homogenity test, $P \leq 0.05$).

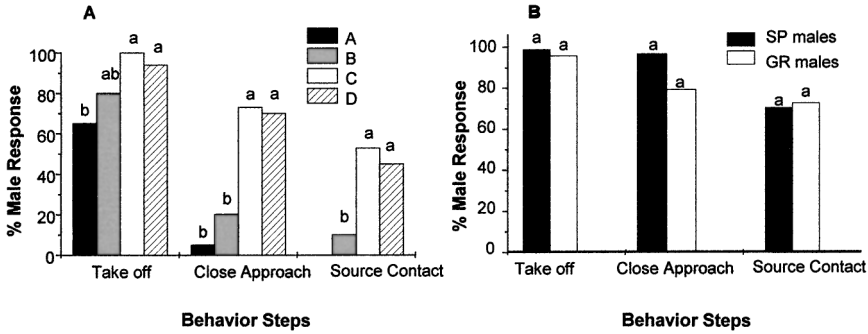


FIG. 1. Percentage of response of male *Sesamia nonagrioides* of three geographic populations (French, Spanish, and Greek) to 1 mg of various combinations of the synthetic pheromone components: **(A)** French males responded to A = **1**; B = **1** + **2** in 90:10; C = **1** + **2** + **3** in 90:10:5; D = **1** + **2** + **3** + **4** in (69:8:8:15) ($N = 20$); **(B)** Spanish and Greek males responded to **1** + **2** + **3** in 84:15:1 ratio ($N = 15$).

Male Response to Synthetic Pheromone Components. The effect of various combinations of the synthetic pheromone components on males' behavior from the three populations was also studied. French males showed a low response when exposed to component **1**. The addition of component **2** at a ratio of 90:10 induced more males to orient to the source, with a low percentage landing on the source. The three-component blend (**1** + **2** + **3**), in a 90:10:5 ratio, elicited a maximal male response, while the four-component blend of **1** + **2** + **3** + **4** in a ratio of 69:8:8:15 did not improve male attraction (Figure 1A). The response of Greek and Spanish males to the three-component pheromone blend (**1** + **2** + **3**) in an 84:15:1 ratio, was similar. (Figure 1B).

Identification of Pheromone Components in Gland Extracts of Females of Three Populations. The components **1**, **2**, and 16:Ac were present in all pheromone gland extracts of the three populations in variable ratios, when the data produced by the three groups were compared. The variance among the three populations was lower when the extracts were analyzed under the same conditions by one specific group (Table 2). Aldehyde **3** was detected in minor amounts directly by GC-MS on relatively concentrated gland extracts obtained from French and Greek origin females. Moreover, 12:Ac was detected in all three populations, and in some of the analyses, 14:Ac was present.

GC and GC-MS analyses using the SPME technique revealed the presence of **1**, **2**, and the saturated 16:Ac with small differences among the three populations. The ratio of the three components (**1**, **2**, and 16:Ac) identified from French, Spanish, and Greek females was 96:3:1, 96.5:1.5:2, and 95.5:3:1.5, respectively.

TABLE 2. COMPOSITION OF FEMALE SEX PHEROMONE OF *Sesamia nonagrioides* IN GLAND EXTRACTS FROM CALLING FEMALES OF FRENCH (FR), SPANISH (SP) AND GREEK (GR) POPULATIONS, MEANS OF 5 REPLICATIONS

	Research groups					
	French[a]		Spanish		Greek	
	Female origin		Female origin[b]		Female origin	
Components	FR	SP	SP	FR	SP	GR
*Z*11–16:Ac (**1**)	85	93	80.9 ± 3.0	80.1 ± 3.2	83.8 ± 2.9	75.5 ± 3.4
*Z*11–16:OH (**2**)	11	3	14.8 ± 2.0	9.8 ± 1.3	6.0 ± 1.4	5.5 ± 0.9
*Z*11–16:Ald (**3**)	ND[c]	ND	ND	0.8 ± 0.4	ND	1.0 ± 0.2
12:Ac (**4**)	ND	ND	ND	7.1 ± 1.3	7.9 ± 1.1	15.5 ± 2.0
16:Ac	4	4	4.2 ± 2.0	2.2 ± 0.7	2.3 ± 0.8	2.5 ± 0.4

[a] SE of the mean, not available.
[b] Analyses by GC-MS of the extracts of Spanish female glands previously treated with PBAN gave the following composition: *Z*11–16:Ac 86.1, *Z*11–16:OH 11.3, *Z*11–16:Ald 2.6.
[c] Not detected (limit of detection was 200 pg).

Field Trials with Synthetic Pheromone Blends. Field experiments were conducted in each country to assess male response to two pheromone blends: A, $\mathbf{1} + \mathbf{2} + \mathbf{3} + \mathbf{4}$ in a 69:8:8:15 ratio and B, $\mathbf{1} + \mathbf{2} + \mathbf{3} + \mathbf{4}$ in a 77:8:10:5 ratio dispensed on rubber septa. In all countries, traps were set up from early May to the middle of October 1998, so that the two generations per year of the insect in France and the three generations per year in Spain and Greece were covered. The mean numbers of moths trapped in each generation and each country for the two-pheromone blends were not significantly different (Table 3).

TABLE 3. MEAN NUMBER OF *Sesamia nonagrioides* MALES CAUGHT PER WEEK IN TRAPS BAITED WITH RUBBER SEPTA LOADED WITH 100 mg OF TWO SYNTHETIC SEX PHEROMONE BLENDS

	Blends[a]					
	Greece		Spain		France	
Generation	A	B	A	B	A	B
1st	1.5^a	1.8^a	2.1^a	2.3^a	1.2^a	1.7^a
2nd	9.7^a	12.2^a	10.1^a	10.1^a	7.1^a	6.8^a
3rd	107.4^a	133.8^a	59.8^a	44.2^a		

[a] A: 69:8:8:15 and B: 77:8:10:5 for $\mathbf{1} + \mathbf{2} + \mathbf{3} + \mathbf{4}$. The comparative field studies were conducted in France, Spain, and Greece, during 1998. Means followed with the same letter in each row and for each country are not significantly different (*Paired t test*, $P \leq 0.05$).

DISCUSSION

The components *Z*11–16:Ac, *Z*11–16:OH, and 16:Ac were unambiguously detected in all French, Spanish, and Greek extracts. However, analyses of the pheromone gland extracts from females of the different populations revealed differences regarding the occurrence of certain minor pheromone components, as well as the ratio of the chemicals present in the blend. The presence of *Z*11–16:Ald in trace quantities was detected by GC-MS in Greek and French origin female extracts. The detection of *Z*11–16:Ald in trace quantities, after treatment of the pheromone gland with PBAN and subsequent derivatization with pentafluorobenzyl hydroxylamine hydrochloride (Mas et al., 2000) and by SPME headspace volatiles collected from French female pheromone glands incubated in physiological solution (Améline, 1999), has been described elsewhere. The presence of this compound in small quantities and its possible biosynthesis during release makes its detection and quantification difficult and sometimes ambiguous. The fourth component of the blend, 12:Ac, was detected only by one group, and in some cases, 14:Ac was detected in gland extracts and SPME collections. 12:Ac, 14:Ac, and 16:Ac were not attractive to males when tested individually; neither did they improve male attractiveness when added to the blend (Ameline, 1999). No other minor active components were detected.

Minor differences in the ratio of the two major components *Z*11–16:Ac and *Z*11–16:OH were also observed. Studies on pheromone components of other lepidopteran species demonstrated high levels of repeatability in the ratio of the compounds emitted by individual females for geometric isomers, while the variance for other components was high (Barrer et al., 1987; Witzgall and Frérot, 1989).

Wind-tunnel experiments revealed no significant difference of male responses to various pheromone sources despite the different origin of male. The results with the synthetic pheromone components revealed that *Z*11–16:Ald is essential for male attraction, while 12:Ac does not increase male attraction. These results agree with those reported by Sans et al. (1997), in that 12:Ac is not essential for male response in wind tunnel experiments. In this context, single-sensillum studies have revealed the existence of different receptor cells tuned to compounds **1**, **2**, and **3**, but not **4** (Guerrero et al., in preparation).

Furthermore, wind-tunnel and field experiments indicated that males of the three populations responded equally well to a relatively broad range of pheromone blends, suggesting that the *S. nonagrioides* male response window is wider than the variability observed in female pheromone production. Similar male response has been reported for other moths (Haynes and Baker, 1988; Löfstedt, 1990).

Variation in the pheromone composition of several lepidopteran species from different geographic populations is well documented (Cardé and Baker, 1984; Phelan, 1993). In our case, although some variations in the pheromone composition between the three populations of *S. nonagrioides* were detected, the

wind-tunnel and field results do not support the assumption of the existence of different geographic pheromone types of the corn stalk borer. Our combined data suggest that new and improved formulations should be developed for monitoring or mating disruption experiments, particularly focusing on improving the stability and slow release of the minor pheromone components.

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SEX ATTRACTANTS FOR MOTHS OF VIETNAM: FIELD ATTRACTION BY SYNTHETIC LURES BAITED WITH KNOWN LEPIDOPTERAN PHEROMONES

TRAN VAN HAI,[1] LE VAN VANG,[1] PHAM KIM SON,[1] SHIN-ICHI INOMATA,[2] and TETSU ANDO[2,*]

[1]*Department of Plant Protection*
College of Agriculture, Cantho University
Cantho City, Vietnam

[2]*Graduate School of Bio-Applications and Systems Engineering*
Tokyo University of Agriculture and Technology
Koganei, Tokyo 184-8588, Japan

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Abstract—Screening tests of synthetic lepidopteran sex pheromones were carried out at orchards in the Mekong Delta over an approximately two-year period starting from December 1998. Monoenyl acetates with a C_{10}–C_{14} chain attracted six species distributed mainly in Southeast Asia: *Adoxophyes privatana*, *Archips atrolucens*, and *Meridemis furtiva* in the Tortricidae family, and *Argyrogramma signata*, *Spodoptera pectinicornis*, and *Zonoplusia ochreata* in the Noctuidae family. These were in addition to three other noctuid species that had been attracted during previous field examinations within a temperate zone. Furthermore, male moths of three species belonging to the Cosmopterigidae, Gelechiidae, or Batrachedridae family were also caught by traps baited with acetates. Trienes with a C_{18}–C_{21} chain and their monoepoxides, which are stereotypes of pheromones secreted by females in the Geometridae family, failed to attract any geometrid male, but attracted three Noctuidae species and four Arctiidae species.

Key Words—*Adoxophyes privatana, Archips atrolucens, Meridemis furtiva, Argyrogramma signata, Spodoptera pectinicornis, Zonoplusia ochreata*, Tortricidae, Noctuidae, Plusiinae, Arctiidae.

* To whom correspondence should be addressed. E-mail: antetsu@cc.tuat.ac.jp

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INTRODUCTION

Sex pheromones have been identified in female moths of about 500 species (Arn et al., 1992, 1997). Some are used as tools for integrated pest management (Cardé and Minks, 1995; Wakamura, 1992). The largest chemical group of lepidopteran pheromones is composed of unsaturated C_{10}–C_{18} straight-chain compounds with a functional group at the terminal position (type I), and they are biosynthesized from saturated fatty acids. The pheromones of the second group consist of unbranched C_{17}–C_{23} (3*Z*,6*Z*,9*Z*)-trienes, (6*Z*,9*Z*)-dienes, and their monoepoxy derivatives (type II), which lack a terminal functional group and are probably biosynthesized from linolenic acid and linoleic acid (Millar, 2000). Additionally, field screening of the synthetic pheromones and their analogs has revealed specific male attractants of more than 1200 lepidopterous species (Arn et al., 1992, 1997). Most of these species are cosmopolitan or are distributed within a temperate zone, and knowledge of the sex pheromones and the attractants of tropical species is limited. We are interested in developing sex pheromones of tropical species for a new pest control program in Vietnam. We started screening tests of chemicals in both types I and II at the end of 1998 in order to gain information on the mating communication systems of the tropical species and to compare the results in Southeast Asia with those of past field tests, particularly with a survey conducted in Japan. This paper deals with two-year tests conducted at orchards in the Mekong Delta. The results are informative for pheromone research in Vietnam, since specific attraction for males of 19 species was found, including some moths from groups in which no sex pheromones or sex attractants have been identified.

METHODS AND MATERIALS

Chemicals. The chemical structures of synthetic lures are abbreviated as follows; *Z* = *Z* double bond, *E* = *E* double bond, epo = *cis*-epoxy ring, number before hyphen = position of a double bond or epoxy ring, number after hyphen = carbon number of a straight chain, OAc = acetate, OH = alcohol, Ald = aldehyde, and H = hydrocarbon. Seven monoenyl acetates (*Z*5–10:OAc, *Z*5–12:OAc, *Z*7–12:OAc, *Z*9–12:OAc, *Z*9–14:OAc, *Z*11–14:OAc, and *E*11–14:OAc), three monoenyl alcohols (*Z*7–12:OH, *Z*11–14:OH, and *E*11–14:OH), and two dienyl acetates (*Z*9,*E*11–14:OAc and *Z*9,*E*12–14:OAc) with >97% purity were supplied by Shin-etsu Chem. Co., Ltd. (Tokyo, Japan). Monoenyl aldehyde (*Z*11–14:Ald) was prepared from *Z*11–14:OH by oxidation with pyridinium chlorochromate. Four trienes (*Z*3,*Z*6,*Z*9–18:H, *Z*3,*Z*6,*Z*9–19:H, *Z*3,*Z*6,*Z*9–20:H, and *Z*3,*Z*6,*Z*9–21:H) and 12 epoxydienes (racemic mixtures of epo3,*Z*6,*Z*9–18:H, *Z*3,epo6,*Z*9–18:H, *Z*3,*Z*6,epo9–18:H, and their C_{19}–C_{21} homologs) with >95% purity were synthesized from linolenic acid as previously described (Ando et al., 1993).

Field Tests. One milligram of each chemical (single-component lure) or one milligram of a mixture of two chemicals (two-component lure) dissolved in *n*-hexane (about 100 μl) was applied to a white rubber septum (8 mm OD, Aldrich Chemical Co., Ltd.), which was not specially coated with any antioxidants or stabilizers. After vaporization of the solvent, the septum was placed in a sticky trap (30 $\times$ 27 cm bottom plate with a roof, Takeda Chemical Ind., Ltd., Osaka, Japan). Each lure was replicated twice, and four extra traps with a septum including no chemicals were used as control. Traps were set 1.5 m above the ground.

In Cantho City, Vietnam, screening tests were carried out at orchards of Chinese apple, guava, and logan from December 1998 to November 1999 (test 1), and at other orchards of plum and guava from January to December in 2000 (test 2). Each lure was replaced every two months. Captured moths were counted once a week.

The activity of the following 28 lures was examined in test 1; *Z*7–12:OAc and its mixtures with *Z*9–14:OAc (9:1, 5:5, and 1:9), *Z*9–14:OAc and its mixtures with *Z*11–14:OAc (9:1, 5:5, 1:9, and 0:10), *E*11–14:OAc and its mixtures with *Z*11–14:OAc (9:1, 5:5, and 1:9), *Z*11–14:OH and its mixture with *Z*11–14:OAc (5:5), *E*11–14:OH, *Z*9,*E*11–14:OAc and its mixture with *Z*9–14:OAc (5:5), *Z*9,*E*12–14:OAc and its mixture with *Z*9–14:OAc (5:5), *Z*3,*Z*6,*Z*9–18:H, *Z*3,*Z*6,*Z*9–19:H, and each of their monoepoxy derivatives. In test 2, the activity of the following 28 lures was examined; *Z*5–10:OAc and its mixtures with *Z*7–12:OAc (9:1, 5:5, 1:9, and 0:10), *Z*5–12:OAc and its mixtures with *Z*7–12:OAc (9:1, 5:5, and 1:9), *Z*9–12:OAc and its mixtures with *Z*7–12:OAc (9:1, 5:5, and 1:9), *Z*7–12:OH and its mixtures with *Z*7–12:OAc (9:1, 5:5, and 1:9), *Z*11–14:Ald and its mixtures with *Z*11–14:OAc (5:5 and 0:10), *Z*3,*Z*6,*Z*9–20:H, *Z*3,*Z*6,*Z*9–21:H, and each of their monoepoxy derivatives.

Identification of Insects. Some parts of attracted moths were sent to Japan for species identification by Prof. Toshiya Hirowatari (species in the families Tortricidae, Cosmopterigidae, Gelechiidae, and Batrachedridae), Dr. Shigero Sugi (species in the families Noctuidae and Arctiidae), and Dr. Masashi Nomura (species in the subfamily Plusiinae).

RESULTS AND DISCUSSION

Overview of Discovered Attractants. For field trials in the first year (test 1), chemicals with a C_{14} chain were exclusively selected as lures among pheromone components in type I in order to attract tortricid and noctuid males. These compounds have been frequently identified from pheromone glands of the females (Arn et al., 1992, 1997). Since lepidopteran pheromones are generally composed of multiple components (Arn et al., 1992, 1997), we made some two-component lures in addition to single-component lures. The mixing ratio was limited to one

(5:5) or three (1:9, 5:5, and 9:1) to reduce the total number of traps. In test 1, *Z*7–12:OAc, only one chemical with a different chain length attracted several species. Based on this result, attraction by the mixtures including *Z*7–12:OAc as a common component was mainly evaluated in the next year (test 2). Among the chemicals in type II, trienes and epoxydinenes with a C_{18}–C_{21} chain were systematically evaluated for their activity in test 1 and test 2 without mixing with each other.

The field tests at orchards in the Mekong Delta successfully revealed the species-specific male attraction of 19 lepidopteran species. Table 1 shows the attractants, the main flight seasons, and the total number of captured moths. While the sex pheromones of two species and an attractant of one species have already been reported, attractants of six taxonomically identified species, and probably those of 10 other taxonomically unidentified species, were first found in this study.

Attraction of Tortricid Species. Tetradecenyl compounds unsaturated at the 11 position have been identified as key pheromone components of many species in the Tortricidae family (Arn et al., 1992, 1997). Our field tests showed the attraction of three tortricid species by *Z*11–14:OAc and its mixtures (Table 1). Among the tested lures, the best attractant for *Adoxophyes privatana* was a 9:1 mixture of *Z*11–14:OAc and *Z*9–14:OAc and that for *Archips atrolucens* was a 5:5 mixture of *Z*11–14:OAc and *E*11–14:OAc, while *Meridemis furtiva* was most strongly attracted by traps baited only with *Z*11–14:OAc. These three species have been recorded in Southeast Asia, but not in Japan (Inoue et al., 1982). *A. privatana* is a species closely related to *Adoxophyes honmai* and *Adoxophyes orana*, pests of tea and fruit trees. The *A. privatana* males were caught by the lure including the same monoenyl acetates as pheromones of the other two species (Tamaki et al., 1971a,b; Meijer et al., 1972), but the composition ratio was quite different from those of the latter pheromones. In the genus *Archips*, sex pheromones have been identified in seven species, and sex attractants have been discovered for 17 species (Arn et al., 1992, 1997). For the specific attraction of these species, *Z*11–14:OAc is an important common component. This characteristic was also indicated in the tropical *Archips* species, *A. atrolucens*. The attraction of males in the genus *Meridemis* has not been reported previously. In this screening, specific attraction was not found for the lures including *Z*11–14:OH, *E*11–14:OH, and *Z*11–14:Ald.

Attraction of Other Microlepidopteran Species. The traps baited with *Z*11–14:OAc also captured many males of a species in the Cosmopterigidae family. Because the taxonomy of microlepidopteran insects in Southeast Asia has not been sufficiently established, the genus of this species has not yet been identified. Another unidentified gelechiid species was specifically attracted to *Z*9–14:OAc, and a 9:1 mixture of *Z*7–12:OAc and *Z*9–14:OAc was the best attractant for *Batrachedra* sp. (Batrachedridae, Table 1).

Attraction of Noctuid Species by Type I Pheromones. Monoenyl acetates attracted six species in the Noctuidae family (Table 1). Attraction of the following

TABLE 1. SEX ATTRACTANTS FOUND BY RANDOM SCREENING TESTS IN CANTHO CITY (VIETNAM), DECEMBER 1998–DECEMBER 2000

Family and species	Attractant	Main season of flight and total males captured (N)[a]
Tortricidae		
Adoxophyes privatana Walker[b]	*Z*11–14:OAc + *Z*9–14:OAc (9:1)	March–April (435)*, June (69)*, Nov. (26)*
Archips atrolucens Diakonoff[b]	*Z*11–14:OAc + *E*11–14:OAc (5:5)	Dec.–March (21)*, May–June (13)*
Meridemis furtiva Diakonoff[b]	*Z*11–14:OAc	March–June (223)*
Cosmopterigidae		
Gen. et sp.	*Z*11–14:OAc	March–June (354)*
Gelechiidae		
Gen. et sp.	*Z*9–14:OAc	Jan.–March (234)*
Batrachedridae		
Batrachedra sp.	*Z*7–12:OAc + *Z*9–14:OAc (9:1)	Jan.–April (145)*, June (18)*, Aug.–Sept. (40)*
Noctuidae		
Argyrogramma signata F.[c]	*Z*5–10:OAc	Jan.–March (64)**, June–July (21)**, Oct. (25)**
Chrysodeixis eriosoma Doubleday[c]	*Z*7–12:OAc	Sept.–Nov. (23)*
	*Z*7–12:OAc + *Z*9–14:OAc (9:1)	Dec.–Jan.(8)*
	*Z*7–12:OAc + *Z*9–12:OAc (9:1)	March (14)**, Oct.–Nov. (70)**
Ctenoplusia agnata Staudinger[c]	*Z*7–12:OAc	Jan.–Apr. (251)**, Aug.–Dec. (424)**
Ctenoplusia albostriata Bremer & Grey[c]	*Z*7–12:OAc + *Z*5–10:OAc (9:1)	Jan.–March (152)**, Oct.–Nov. (16)**
Zonoplusia ochreata Walker[c]	*Z*7–12:OAc + *Z*5–12:OAc (5:5)	Feb.–May (73)**, Aug.–Dec. (97)**
Spodoptera pectinicornis Hampson[d]	*Z*7–12:OAc	June–Oct. (51)*
Hypena sp.[e]	*Z*3,*Z*6,epo9–21:H	Jan.–Feb. (28)**
Luceria sp.[f]	*Z*3,*Z*6,epo9–18:H	Dec.–Feb. (140)*, July–Sept. (58)*
Zanclognatha sp.[g]	*Z*3,epo6,*Z*9–19:H	Dec.–Jan. (29)*, Aug.–Sept. (38)*
Arctiidae		
Cyana sp.[h]	*Z*3,*Z*6,epo9–18:H	Aug. (31)*
Schistophleps sp. 1[h]	Z3,Z6,epo9–19:H	Dec.–March (106)*, June–July (14)*
Schistophleps sp. 2[h]	Z3,Z6,Z9–19:H	Dec.–April (254)*, Aug.–Nov. (65)*
Schistophleps sp. 3[h]	Z3,Z6,Z9–21:H	Feb.–Sept. (242)**

[a] Captured males in test 1 from December 1998 to November 1999 (*), and in test 2 from January to December 2000 (**).
[b] Tortricinae.
[c] Plusiinae.
[d] Amphipyrinae.
[e] Hypeninae.
[f] Ophiderinae.
[g] Herminiinae.
[h] Lithosiinae.

three species was also achieved: *Argyrogramma signata* (Plusiinae) by *Z*5–10:OAc, *Zonoplusia ochreata* (Plusiinae) by a 5:5 mixture of *Z*7–12:OAc and *Z*5–12:OAc, and *Spodoptera pectinicornis* (Amphipyrinae) by *Z*7–12:OAc. While *Z. ochreata* is also distributed in Japan, the other two are tropical species (Inoue et al., 1982). Additionally, this screening showed the male attraction of three other noctuid species in the Plusiinae subfamily: *Chrysodeixis eriosoma*, *Ctenoplusia agnata*, and *Ctenoplusia albostriata*. Their larvae are defoliators of vegetables (Inoue et al., 1982), and their sexual communication has been reported. Sex pheromones of many Plusiinae species include *Z*7–12:OAc as a key component (Arn et al., 1992, 1997). Table 2 shows the results of field attraction in the Mekong Delta by *Z*7–12:OAc and its mixtures with related compounds. The sex pheromone of *C. eriosoma* is composed of *Z*7–12:OAc and *Z*9–12:OAc (Benn et al., 1982), and the 9:1 mixture was the best attractant in this test. *C. agnata* in Vietnam was attracted most strongly by the single-component lure of *Z*7–12:OAc, whereas *Z*7–12:OAc and *Z*9–12:OAc have been previously identified in this species, and the 10:1 mixture was the best lure in Japan (Sugie et al., 1990). The same mixture in a 9:1 ratio caught few males in this test, indicating that the Vietnamese and Japanese strains have different systems for sexual communication. The highly specific attraction of *C. albostriata* attained by a mixture of *Z*7–12:OAc and *Z*5–10:OAc in Vietnam coincided well with the results of field tests in Japan (Inomata et al., 2000). The differences in optimum attractants shown in Table 2 seemed to be sufficient to prevent the cross-attraction of heterospecific males.

Figure 1 shows the monthly data of captured males of three Plusiinae species in test 2: *C. eriosoma*, *C. agnata*, and *C. albostriata*. The monthly average temperature in Cantho City is almost constant, around 27°C throughout the year, but there are two seasons based on rainfall. The dry season, from January to March, is suitable for the cultivation of vegetables. The rainy season starts in April, when many vegetables fields are changed to paddy fields. The above-mentioned species do not have a diapause system, and it was expected that continuous and overlapping generations would occur throughout the year. However, male catches from April to July were rarely observed. Their flights were observed primarily in the dry season and, interestingly, in the latter half of the rainy season. We plan to examine the ecological aspects of Plusiinae pests in detail using pheromone traps.

Attraction of Noctuid Species by Type II Pheromones. Field screening with C_{18}–C_{21} chain compounds in type II showed the attraction of three more Noctuidae species: *Hypena* sp. by *Z*3,*Z*6,epo9–21:H; *Luceria* sp. by *Z*3,*Z*6,epo9–18:H; and *Zanclognatha* sp. by *Z*3,epo6,*Z*9–19:H (Table 1). Although only genera were determined for these species, sex pheromones and attractants have not been reported for any other species in the former two genera. Among the *Zanclognatha* species, it has been reported that males of *Z. lunaris* were attracted to disparlure, a methyl-branched epoxyalkane (Minyailo et al., 1977). They belong to the subfamilies Hypeninae, Ophiderinae, and Herminiinae, which are recognized as

TABLE 2. MALE ATTRACTION OF FOUR PLUSIINAE SPECIES BY SYNTHETIC LURES BAITED WITH *Z*7–12:OAC AND MIXTURES WITH ITS RELATED COMPOUNDS, CANTHO CITY (VIETNAM), 2000[a]

*Z*7–12:OAc Lure (mg)	Related comp. (mg)		Total captured males for one year and (number/trap/night at the indicated period)[b]			
			C. eriosoma	*C. agnata*	*C. albostriata*	*Z. ochreata*
1.0	none		4 (0.02 c)	682 (3.83 a)	1 (0)	0 (0)
0.9	Z5-10:OAc	0.1	0 (0)	24 (0)	173 (1.86 a)	0 (0)
0.5		0.5	5 (0.05 c)	4 (0)	48 (0.38 b)	0 (0)
0.1		0.9	25 (0.07 c)	1 (0)	1 (0.02 c)	0 (0)
0		1.0	0 (0)	0 (0)	0 (0)	0 (0)
0.9	Z7-12:OH	0.1	0 (0)	41 (0.19 b)	1 (0)	0 (0)
0.5		0.5	1 (0)	33 (0.07 b)	1 (0)	0 (0)
0.1		0.9	0 (0)	0 (0)	0 (0)	0 (0)
0.9	Z5-12:OAc	0.1	1 (0)	13 (0.10 b)	0 (0)	4 (0.02 b)
0.5		0.5	0 (0)	0 (0)	0 (0)	199 (0.57 a)
0.1		0.9	0 (0)	0 (0)	0 (0)	0 (0)
0.9	Z9-12:OAc	0.1	87 (1.67 a)	3 (0.02 b)	0 (0)	0 (0)
0.5		0.5	24 (0.29 b)	2 (0)	0 (0)	0 (0)
0.1		0.9	0 (0)	0 (0)	0 (0)	0 (0)
control			0 (0)	0 (0)	0 (0)	0 (0)

[a] Each lure was replicated twice using two traps except for four traps of control.
[b] Values were calculated with the data from October 11 to November 1 for *C. eriosoma, C. agnata,* and *Z. ochreata,* and from January 12 to February 2 for *C. albostriata.* Numbers within each column followed by a different letter are significantly different at $P < 0.05$ by Duncan's multiple-range test.

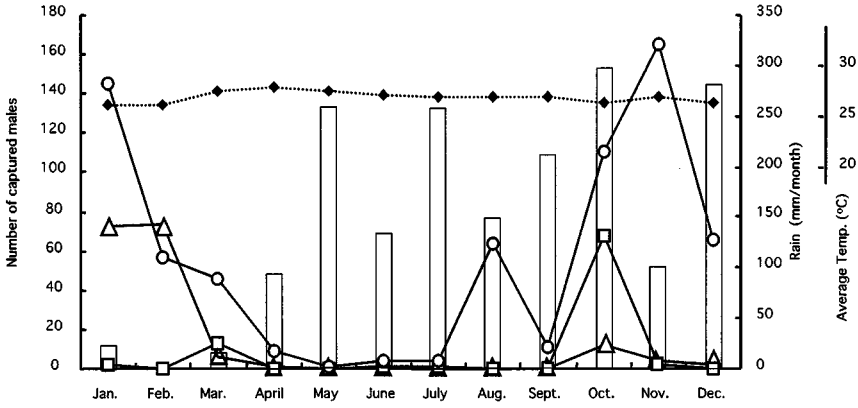


FIG. 1. Monitoring of three Plusiinae species by pheromone traps in orchards in the Mekong Delta (Cantho City, Vietnam) in 2000. Monthly totals, which were captured by two traps baited with the following lures, are shown; *Chrysodeixis eriosoma* by the 9:1 mixture of Z7–12:OAc and Z9–12:OAc (□), *Ctenoplusia agnata* by Z7–12:OAc (○), and *Ctenoplusia albostriata* by the 9:1 mixture of Z7–12:OAc and Z5–10:OAc (△). Temperature and rainfall were indicated by ♦ and open bars, respectively.

Noctuidae groups in Quadrifinae that secrete chemicals of type II as a pheromone component (Ando et al., 1993; Millar, 2000).

Attraction of Arctiid Species. Compounds of type II, which had attracted many species in the Geometridae family in Japan, did not attract any geometrid males in Vietnam. Instead, their field evaluation in Vietnam showed the attraction of four arctiid species (Table 1), whereas no arctiid males had been attracted in the same screening tests in Japan (Ando et al., 1993, 1995). Although their species names are unknown, they belong to the subfamily of Lithosiinae, which includes many tropical species, and *Cyana* and *Schistophleps* were assigned as their genera. Sex pheromones and attractants have not been reported for any species in Lithosiinae (Millar, 2000).

In conclusion, these results indicate the usefulness of field trials with synthetic lures in pheromone research of tropical species. Screening tests with known pheromone components provide leads to possible sex pheromones for other species, not only in the same group, but also in a different group for which sex pheromones have not been reported, as well as information about pheromone differences between geographically isolated populations. Although we captured male moths of some pest species of vegetables, our attempt to attract harmful pests in orchards was not successful. One of the main reasons might be the limited selection of test chemicals and their combination. We are going to evaluate lures, including other synthetic pheromone components, for example, aldehydes and compounds with a

C_{16} chain, in different fields in the Mekong Delta. Pheromone research may allow us to obtain a better understanding of the taxonomy of lepidopteran species from Vietnam.

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OLIGOSACCHARIDE COMPOSITION AND PHEROMONAL ACTIVITY OF MALE TERGAL GLAND SECRETIONS OF THE GERMAN COCKROACH, *Blattella germanica* (L.)

SATOSHI NOJIMA,[1,3] SOICHI KUGIMIYA,[1] RITSUO NISHIDA,[1,*] MASAYUKI SAKUMA,[2] and YASUMASA KUWAHARA[1]

[1]*Laboratory of Chemical Ecology*
Division of Applied Life Sciences
[2]*Laboratory of Insect Physiology*
Division of Applied Biosciences
Graduate School of Agriculture
Kyoto University
Sakyo-ku, Kyoto 606-8502, Japan

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Abstract—In the sequential courtship behavior of the German cockroach, *Blattella germanica* (Dictyoptera: Blattellidae), females feed on the tergal gland secretions from the male tergites. The phagostimulative components in the male secretions were composed of a complex mixture of oligosaccharides and phospholipids. Besides seven oligosaccharides previously identified, two new trisaccharides, *O*-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl α-D-glucopyranoside and *O*-α-glucopyranosyl-(1→4)-*O*-α-glucopyranosyl-(1→1)-*myo*-inositol, were characterized as the sugar components of the secretions. The activity of the sugar components was compared in combination with the lipid fraction. Individual oligosaccharides exhibited the activity either alone or synergistically with the phospholipids. A complex mixture of these primary substances in the tergal gland secretions, thus, serves as a pheromonal phagostimulant in the precopulatory behavior, strongly eliciting feeding response in the female cockroach.

Key Words—*Blattella germanica*, German cockroach, tergal gland, male courtship pheromone, phagostimulant, oligosaccharides, phospholipids.

* To whom correspondence should be addressed. E-mail: ritz@kais.kyoto-u.ac.jp
[3] Present address: Department of Entomology, New York State Agricultural Experiment Station, Cornell University, Geneva, New York 14456.

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INTRODUCTION

The courtship behavior of the German cockroach, *Blattella germanica* (L.), has been studied by many researchers, and the involvement of chemical cues in the sequential steps of precopulatory behavior has been suggested (Sikord, 1918; Wille, 1920; Gould and Deay, 1937; Roth and Willis, 1952, 1954; Ishii, 1972; Liang and Schal, 1993). In an encounter, the male touches the female with his antennae and raises his wings upward in response to a contact chemical factor on the female cuticular surface (female sex pheromone) (Nishida and Fukami, 1983; Schal et al., 1990). The male simultaneously turns around 180° and displays his abdominal tergal glands to the female. While the female feeds on the tergal secretions (male sex pheromone), the male pushes his abdomen under the female and clasps her genitalia with his left phallomere to accomplish genital connection. Although the male secretions have been chemically analyzed in the past (Brossut et al., 1975; Sreng, 1979), the pheromonally active compounds in the secretions have not been known until recently. We have characterized a series of oligosaccharides and polar lipids as the two main classes of male courtship pheromone components in the tergal glands (Nojima et al., 1999a). Further investigation of the secretions led to the isolation of additional oligosaccharide components. We describe here the structural elucidation of these compounds, and overview the chemical composition and pheromonal activity of the sugar components.

METHODS AND MATERIALS

Insects. Blattella germanica (L.) were raised under conditions described in a previous paper (Nojima et al., 1996). Newly emerged adults were reared separately, and sexually mature unmated males and females were used for tergal gland extraction and behavioral bioassay, respectively.

Phagostimulant Assays. The polyethylene glycol (PEG) film method was employed for the phagostimulant assay (Nojima et al., 1996). A test sample (in male equivalency) was applied to a micro cover glass plate as a mixture with 40 μg of PEG 70,000 (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The test female (6 days old) was allowed to contact the film for 1 hr. The activity of a test material was recorded as the percentage of PEG films with bite marks (Nojima et al., 1996). For the qualitative assays, each sample was tested using 60–90 insects, and the results were analyzed by Fisher's exact probability followed by the sequential Bonferroni test (Rice, 1989). For the quantitative assays, expressed by the male equivalent per 40 μg PEG spot (ME/PEG), the median effective concentrations (EC_{50}) of active materials were evaluated by probit analysis on the correlation between concentration of the material and phagostimulative responses (Sakuma, 1998).

Spectral Instrumentation. Optical rotations were measured with a Jasco DIP-370 digital polarimeter. High-resolution fast atom bombardment (FAB) mass spectra were recorded with a Jeol HX221A mass spectrometer using dithiothreitol and thioglycerol matrix for a positive mode and triethanolamine matrix for a negative mode. ^{1}H and ^{13}C NMR spectra including the H-H COSY, C-H COSY, and HMBC spectra were measured with a Bruker ARX-500 spectrometer (^{1}H, 500 MHz; ^{13}C, 125 MHz) in D_2O. The chemical shifts were referenced to 3-(trimethylsilyl)-propionic acid sodium salt for D_2O (acetone was used as a secondary internal standard in D_2O: $^{1}H = 2.23$ ppm and $^{13}C = 33.07$ ppm at 27°C).

Extraction and Fractionation of Pheromone Components. Pheromone components were extracted from the eighth tergal glands (TG-8) by the method previously described (Nojima et al., 1999a). A portion of the phagostimulative methanol layer was fractionated by a reverse-phase column (Sep-Pak Vac cartridge C18, 10 g; Waters Associates), eluting in a sequence with 150 ml each of water-methanol-formic acid in ratios of 100:0:1, 40:60:1, 20:80:1, and 0:100:0 (v/v). The water eluate was fractionated into 16 subfractions by preparative HPLC with two Radial Pak cartridges (100 × 8 mm ID, packed with μ-Bondapak NH_2; Waters) eluted with 36% water in acetonitrile (2.0 ml/min) (Figure 1A).

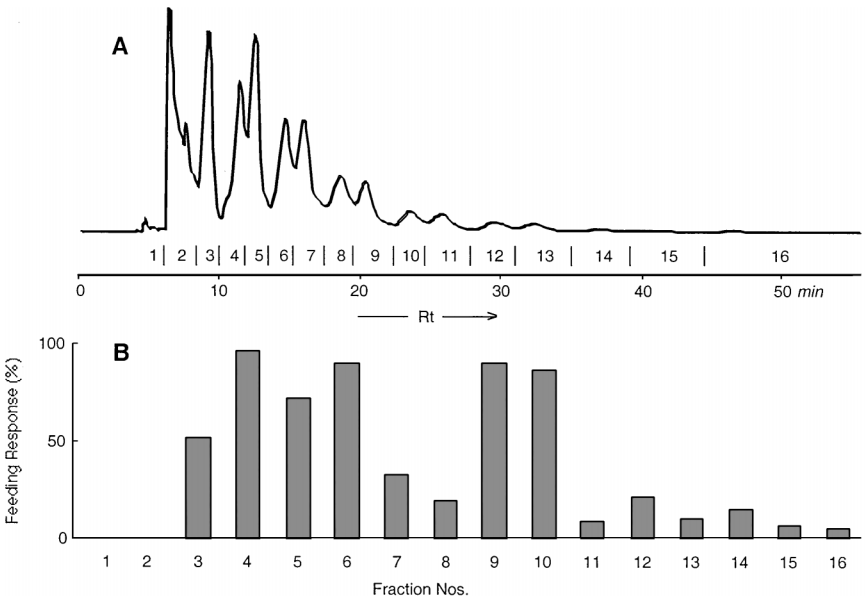


FIG. 1. (A) Liquid chromatogram of the sugar fraction on an aminopropylsilyl bonded silica gel column eluted with 36% water in acetonitrile, 2.0 ml/min, refractive index detection. (B) Feeding response of the virgin females of *Blattella germanica* to each sugar subfraction at a concentration of 1.0 ME/PEG ($N = 90$).

Chemicals. Maltotriose (**IIIa**) and glucosyl trehalose (**IIIb**) used for phagostimulant assay were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan), respectively. Pure samples of compounds **IIIc** and **IIId** were isolated from S-5 and S-6 (Figure 1A), respectively, by preparative HPLC with an ODS column (YMC PAK ODS A-323, 250 mm × 10 mm ID, YMC Co., Ltd., Kyoto, Japan) eluting with water 1.0 ml/min. The retention times of **IIIc** and **IIId** were 20.6 and 16.8 min, respectively.

RESULTS

Isolation of Oligosaccharides. Pheromonal secretions that induced feeding behavior in female *B. germanica* were extracted from the TG-8 segment of approximately 10,000 male cockroaches (Nojima et al., 1999a). A methanol-soluble fraction (636 mg) was separated into four fractions on a reverse-phase cartridge column, and the activity was recovered from fractions eluted with water (containing 1% formic acid) (sugar fraction, 398 mg) and with methanol (lipid fraction, 202 mg), respectively. A mixture of these two fractions synergistically exhibited phagostimulant activity (EC_{50} 0.0063 ME/PEG) close to that of the crude extract (EC_{50} 0.0047 ME/PEG) (Nojima et al., 1999a). The sugar fraction was subjected to HPLC with an aminopropylsilyl-bonded silica gel column and separated into 16 subfractions (S-1 to S-16) (Figure 1A). S-3, S-4, S-5, S-6, S-9, and S-10 individually exhibited more than 50% response to the females at 1.0 ME/PEG (Figure 1B). The activity of these subfractions was remarkably enhanced by mixing with the lipid fraction in the same equivalency (e.g., S-4 alone: EC_{50} 0.17 ME/PEG; S-4 + lipid fraction: EC_{50} 0.019 ME/PEG; S-5 alone: EC_{50} 0.69 ME/PEG; S-5 + lipid fraction: EC_{50} 0.016 ME/PEG; lipid fraction alone: EC_{50} 0.14 ME/PEG). Nine oligosaccharides with phagostimulant activity were isolated from subfractions S-3, -4, -5, -6, -8, and -10 by means of successive HPLC. Table 1 summarizes the subfractions from which each compound was isolated, $[\alpha]_D$ values, approximate contents (micrograms per gland), and phagostimulant activity of the saccharides isolated from the tergal glands.

Identification of Oligosaccharides. The identity of seven oligosaccharides from male tergal glands was reported in an earlier paper (Nojima et al., 1999a). These compounds were classified into two homologous series: D-maltose (**IIa**), D-maltotriose (**IIIa**) and D-maltotetraose (**IVa**) [maltooligosaccharides], and *O*-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl α-D-glucopyranoside (**IIIb**), *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl α-D-glucopyranoside (**IVb**), *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl α-D-glucopyranoside

TABLE 1. PHYSICAL AND BIOLOGICAL DATA OF OLIGOSACCHARIDES ISOLATED FROM *Blattella germanica* MALE TERGAL SECRETIONS

Compound	HPLC subfraction[a]	$[\alpha]_D$ (c)[b]	Content (μg)/gland[c]	EC_{50} (ME/PEG)[d]
IIa	S-3	+134° (0.22)	0.24	3.5
IIIa	S-4	+137° (0.075)	0.15	2.3
IIIb	S-4	+162° (0.59)	0.81	6.3
IIIc	S-5	+145° (3.70)	4.00	ND
IIId	S-6	+114° (0.40)	0.44	ND
IVa	S-6	+159° (0.068)	0.08	11
IVb	S-6	+144° (0.39)	0.53	19
Vb	S-8	+192° (0.23)	0.23	ND
VIb	S-10	+149° (0.14)	0.17	3.4

[a] HPLC subfraction number given in the chromatogram (Figure 1A).
[b] Optical rotations measured in H_2O (temperature: 28° and 27°C for **IIIa** and **IIIb**, otherwise 30°C).
[c] Approximate quantities estimated from HPLC peak area and/or measurement of actual yields.
[d] Median effective concentration for the pure compounds. ND: not determined due to the low feeding responses.

(**Vb**) and *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl α-D-glucopyranoside (**VIb**) [oligoglucosyl trehaloses with (1→4)-α-D-glycosidic linkages]. The Roman numerals represent the number of hexose units. Additionally, we have isolated two saccharides (**IIIc** and **IIId**) not homologous to the above series. Compound **IIIc** was found to be the most abundant sugar constituent in TG-8 (Table 1).

The molecular formula of **IIIc** was determined to be $C_{18}H_{32}O_{16}$ by FAB-MS (positive m/z 505 $[M+H]^+$, 527 $[M+Na]^+$, 543 $[M+K]^+$; negative m/z 503 $[M-H]^-$) and ^1H and ^{13}C NMR spectral data. From the correlation spectroscopy (H-H COSY and C-H COSY), each NMR signal was assigned as shown in Tables 2 and 3. The chemical shifts and coupling constants of the anomeric carbons and protons indicated the presence of three α-glucopyranose units in compound **IIIc**. Two glucose units were found to form an α,α-trehalose structure, to which another α-glucose was attached, by comparing the NMR spectra with those of α,α-trehalose (Tables 2 and 3). The glucosidic linkage between trehalose and glucose was determined to be positioned at C-6′ by HMBC experiment (a correlation observed between H-1″ and C-6′). Consequently, **IIIc** was identified to be *O*-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl α-D-glucopyranoside (Figure 2). The ^{13}C NMR resonances and optical rotation of **IIIc** agreed with those reported in the literature (Koto et al., 1986; Ajisaka and Fujimoto, 1990; Kurimoto et al., 1997).

Compound **IIId** was found by the accurate mass measurement (HRFAB-MS: negative m/z 503.1604 $[M-H]^-$ (Δ mmu −0.8)] to have the molecular formula $C_{18}H_{32}O_{16}$. From the H-H COSY, H-C COSY, and HMBC spectra, each signal was assigned as shown in Tables 2 and 3. Anomeric protons and carbons were

TABLE 2. ^{1}H NMR SPECTRAL ASSINGMENT OF **IIIc** AND **IIId** AND REFERENCE COMPOUNDS[a]

	IIIc	**IIId**	α,α-Trehalose	*myo*-Inositol
H-1	5.20 d (4)	3.62 dd (3, 10)a	5.20 d (4)	3.53 dd (3, 10)
H-2	3.66 dd (4, 10)	4.22 dd (3, 3)	3.65 dd (4, 10)	4.06 dd (3, 3)
H-3	3.85 dd (10, 10)	3.55 dd (3, 10)a	3.85 dd (10, 10)	3.53 dd (3, 10)
H-4	3.46 dd (10, 10)	3.65 dd (10, 10)b	3.45 dd (10, 10)	3.63 dd (10, 10)
H-5	3.83 ddd (2, 5, 10)	3.32 dd (10, 10)	ND	3.28 dd (10, 10)
H-6	3.76 dd (5, 11)	3.82 dd (10, 10)b	3.76 dd (5, 12)	3.63 dd (10, 10)
	3.86 dd (2, 11)		ND	
H-1′	5.20 d (4)	5.20 d (4)		
H-2′	3.68 dd (4, 10)	3.60 dd (4, 10)		
H-3′	3.85 dd (10, 10)	4.05 dd (10, 10)		
H-4′	3.56 dd (10, 10)	3.65 dd (10, 10)		
H-5′	4.02 ddd (2, 5, 10)	3.97 ddd (2, 6, 10)		
H-6′	3.76 dd (5, 12)	3.81 dd (6, 12)		
	3.97 dd (2, 12)	3.89 dd (2, 12)		
H-1″	4.97 d (4)	5.42 d (4)		
H-2″	3.57 dd (4, 10)	3.59 dd (4, 10)		
H-3″	3.73 dd (10, 10)	3.70 dd (10, 10)		
H-4″	3.43 dd (10, 10)	3.43 dd (10, 10)		
H-5″	3.72 ddd (2, 5, 10)	3.73 ddd (2, 6, 10)		
H-6″	3.77 dd (5, 12)	3.77 dd (6, 12)		
	3.86 dd (2, 12)	3.87 dd (2, 12)		

[a] a, b: δ-values with the same letters are interchangeable. ND: not determined due to high multiplicity and signal overlapping.

found as two doublet proton resonances at δ_H 5.20 (H-1′) and 5.42 (H-1″) ($J_{H\text{-}1,2}$ of both were 4 Hz) and two carbon resonances at δ_C 103.24 (C-1′) and 102.44 (C-1″). The H-H COSY spectrum clearly showed that the two anomeric protons correlated with two sets of six protons, respectively, suggesting two hexose units in **IIId**. The coupling constants of $J_{H\text{-}2,3}$, $J_{H\text{-}3,4}$, and $J_{H\text{-}4,5}$ of both hexosyl residues were large ($J = 10$ Hz). These characteristics, together with the coupling constants of the anomeric protons, implied that both hexoses were α-glucopyranoses. The remaining proton signals were observed in the range δ_H 4.22–3.32. The H-H COSY correlation suggested that the other constituent was a cyclitol. The coupling constants of $J_{H\text{-}1,6}$, $J_{H\text{-}3,4}$, $J_{H\text{-}4,5}$, and $J_{H\text{-}5,6}$ were 10 Hz, whereas $J_{H\text{-}1,2}$ and $J_{H\text{-}2,3}$ were 3 Hz. These data indicated that H-4, H-5, and H-6 methines of the cyclitol were in the axial position and located in the vicinal diaxial geometry, whereas H-1 and H-3 were in the axial position and located in the vicinal axial–equatorial geometry, and H-2 was in the equatorial position and located in the vicinal diaxial geometry. These characteristics implied that the cyclitol was *myo*-inositol. The position of the glucosidic linkages of two glucoses and *myo*-inositol were elucidated by an HMBC spectrum. In the spectrum, anomeric protons H-1″

TABLE 3. ^{13}C NMR SPECTRAL ASSINGMENT OF **IIIc** AND **IIId** AND REFERENCE COMPOUNDS[a]

	IIIc	**IIId**	α,α-Trehalose	*myo*-Inositol
C-1	96.23	82.57a	96.07	73.94
C-2	73.83	74.60	73.88	74.99
C-3	75.37	73.92a	75.36	73.94
C-4	72.49	74.96b	72.54	75.21
C-5	75.04	76.85	74.99	77.16
C-6	63.34	74.74b	63.37	75.21
C-1′	96.17	103.24		
C-2′	73.80	74.44		
C-3′	75.64	76.18		
C-4′	72.48	79.69		
C-5′	73.37	73.72		
C-6′	68.21	63.45		
C-1″	100.52	102.44		
C-2″	74.28	74.56		
C-3″	75.92	75.70		
C-4″	72.35	72.19		
C-5″	74.65	75.54		
C-6″	63.31	63.34		

[a] a, b: δ values with the same letters are interchangeable.

(δ_H 5.42) and H-1′ (δ_H 5.20) were found to correlate with the carbons C-4′ (δ_C 79.69) and C-1 (or C-3) (δ_C 82.57), respectively. These data indicated that two glucosyl residues connected with an α-1,4-glucosidic linkage to constitute a maltose moiety, and the maltosyl residue connected to the C-1 (or C-3) position of *myo*-inositol in an α-glucosidic linkage (Figure 2). This was supported by ^{13}C resonances, which showed that the C-1 (or C-3) of *myo*-inositol residue and the C-4′ of glucose residue in **IIId** were shifted to down field ($\Delta\delta_C = 9$ and 7), compared with those of *myo*-inositol and trehalose, respectively (Table 3). Since the C-1 and C-3 are completely equivalent in the symmetrical structure of *myo*-inositol, it was not possible to determine the bonding position of the *myo*-inositol moiety with the maltosyl residue by the NMR spectroscopy. Thus, compound **IIId** was concluded to be *O*-α-glucopyranosyl-(1→4)-*O*-α-glucopyranosyl-(1→1)-*myo*-inositol (Figure 2). The absolute configuration of **IIId** remains to be clarified.

Phagostimulant Activity. The phagostimulant activity of maltooligosaccharides (**IIa**, **IIIa**, and **IVa**) and oligoglucosyl trehaloses with (1→4)-α-D-glucosidic linkages (**IIIb**, **IVb**, **Vb**, and **VIb**) has been previously reported (Nojima et al., 1999a) (see also Table 1 for male equivalent-based EC_{50}). We compared the activity of four isomeric trisaccharides (**IIIa**, **IIIb**, **IIIc**, and **IIId**) individually and in combination with the lipid fraction (at 0.1 ME/PEG) as shown (Figure 3). Maltotriose (**IIIa**) exhibits distinct activity by itself even without the lipid fraction.

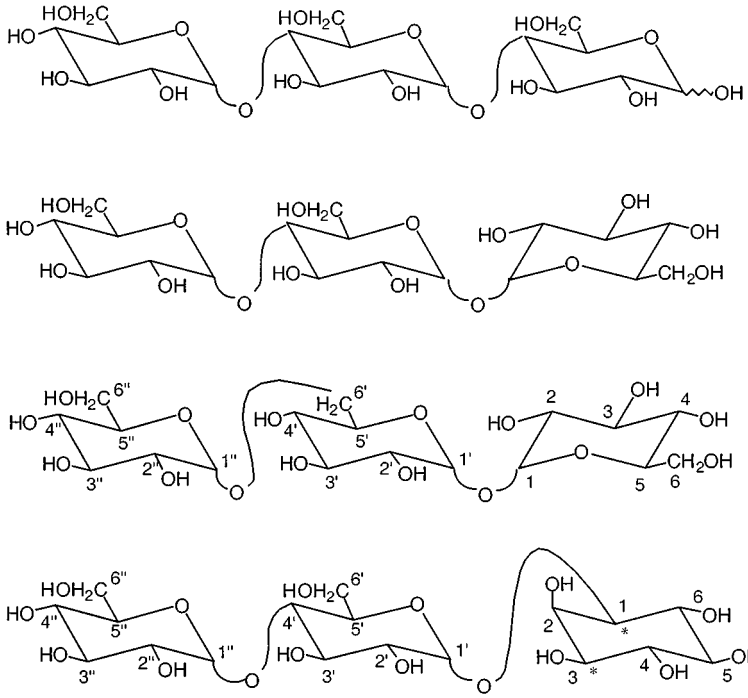


FIG. 2. Chemical structures of phagostimulative oligosaccharides isolated from the 8th tergal glands of *Blattella germanica* males: Maltotriose (**IIIa**), *O*-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl α-D-glucopyranoside (**IIIb**), *O*-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl α-D-glucopyranoside (**IIIc**), *O*-α-glucopyranosyl-(1→4)-*O*-α-glucopyranosyl-(1→1)-*myo*-inositol (**IIId**).

In contrast, **IIIb**, **IIIc**, and **IIId** elicited only weak feeding responses from females, and showed significant activity in a synergistic action with the lipid fraction.

DISCUSSION

The sugar fraction of the tergal secretions was found to consist of glucose oligomers, which serve as the male courtship pheromone, stimulating female feeding responses, particularly when mixed with the phospholipid fraction (Nojima et al., 1999a). The sugar components were classified into three groups: reducing sugars (maltooligosaccharides **IIa**, **IIIa**, **IVa**), nonreducing sugars with (1→4) or (1→6) linkages (oligoglucosyl trehaloses **IIIb**, **IIIc**, **IVb**, **Vb**, **VIb**), and a *myo*-inositol-containing pseudotrisaccharide (**IIId**). Maltotriose (**IIIa**) exhibited the highest activity among the test compounds (Nojima et al., 1999a). There was a tendency for maltooligosaccharides to be more active than the corresponding

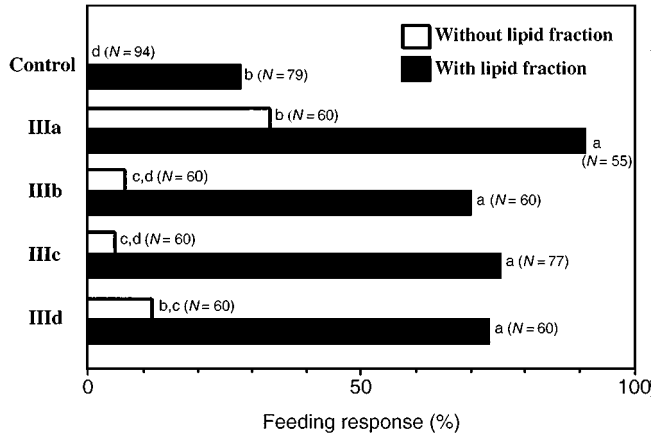


FIG. 3. Phagostimulant activity of trisaccharides (**IIIa**, **IIIb**,**IIIc**, and **IIId**) contained in the male tergal glands of *Blattella germanica* in combination with the phospholipid fraction at a concentration of 0.1 ME/PEG. Different letters indicate statistical differences between samples (Fisher's exact probability tests followed by the sequential Bonferroni test, $P < 0.05$).

oligoglucosyl trehaloses, although **VIb** was a potent phagostimulant among the latter (Table 1). Phospholipids appeared to synergize the activity of individual sugar components greatly, but in varying amplitudes. Although **IIIb**, **IIIc**, and **IIId** elicited only weak feeding responses from females, these compounds showed significant phagostimulant activity in a synergistic action with the lipid fraction (Figure 3). Considering its abundance in the tergal secretions (Table 1), **IIIc** appears to serve as an important sugar component in the courtship pheromone. In addition to these nine oligosaccharides, tergal secretions contained a series of other sugar components, as seen in the liquid chromatogram (Figure 2A). Trehalose (ca. 2 μg/gland) and *myo*-inositol (ca. 1 μg/gland) were found to be prominent components in the sugar subfraction S-3. Trehalose appeared to be a contaminant from the hemolymph, whereas *myo*-inositol was likely from the glands (Nojima et al., 1999a). Other components in the tergal secretions need to be identified in order to clarify the whole profile of the complex oligosaccharide mixture.

Oligoglucosyl trehaloses are known to be produced enzymatically from corresponding maltooligosaccharides by several maltooligosyl trehalose synthases isolated from microorganisms (*Arthrobacter* sp. Q36 and *Sulfolobus acidocaldarius*) (Nakada et al., 1995, 1996). It is interesting that tergal sugars coincidentally include both the substrates as well as rearranged products. However, an involvement of similar enzymes or microbial association in the biosynthesis of the tergal secretions remains to be clarified. Trisaccharide **IIIc** has recently been identified in the body of the silverleaf whitefly, *Bemisia argentifolii*, as a major soluble and possible

storage carbohydrate (Hendrix and Salvucci, 2001). To the best of our knowledge, this is the first report of the *myo*-inositol containing pseudotrisaccharide (**IIId**) isolated in nature.

The counterpart lipid fraction was composed predominantly of phosphatidylcholines (lecithins), and the synergistic activity could be substituted by yolk lecithin (Nojima et al., 1999a). The lipid fraction was found to contain phosphatidylethanolamines as well (unpublished data).

Sugars and lipids are well known as general dietary phagostimulants among cockroaches, including *B. germanica* (Tsuji, 1965, 1966; Nojima et al., 1996). In this pheromone system, the sugar and lipid components showed a synergistic action. Synergistic actions between dietary components are widely known among insects (Dethier, 1972). Male tergal secretions of *B. germanica*, thus, primarily play a behavioral role to induce female feeding behavior, which is basically the same as a general response toward dietary foods (Nojima et al., 1999b).

Male tergal glands are widespread among cockroach species (Roth, 1969). The gland secretions assist in settling the female in an appropriate position for the genital connection in the mating sequence (Roth and Willis, 1952, 1954). The secretions are thought to play various pheromonal roles, such as an attractant, arrestant, phagostimulant, sedative, or excitant (Roth and Dateo, 1966; Roth, 1969; Birch, 1974; Sreng, 1993). Although the tergal substances could serve as an aphrodisiac pheromone (Birch, 1974), the above evidence in the pheromone system of *B. germanica* suggests that the female's persistence in the secretions is merely attributed to the phagostimulant nature of tergal secretions and the ingenious structure of TG-8 (Nojima et al., 1999b). It is a peculiar case of a courtship pheromone presented as a glandular gift inducing feeding behavior in females. Similar nuptial feeding is commonly seen in crickets and often in other species (Engelmann, 1970; Gwynne, 1983, 1997); a beetle, *Neopyrochroa flabellata*, presents cantharidin on the head during courtship in a similar process (Eisner et al., 1996).

The tergal glands of species of the genus *Blattella*, which occur on both seventh and eighth tergites or only on the seventh tergite, are characteristic and rich in variety (Roth, 1969). *B. germanica* males have glands both on the seventh and eighth tergites. Differences in chemical composition of secretions and behavioral roles between these two glandular organs in *B. germanica* need to be clarified. A systematic study of the chemistry and function of secretions in the courtship behavior could provide a key to understanding the evolution of this unique glandular gift widely developed in the Blattaria.

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Book Review

Marine Chemical Ecology. 2001. Edited by James B. McClintock and Bill J. Baker. CRC Press, Boca Raton, Florida. $129.95. 610 pp. ISBN 0-8493-9064-8

This compendium is meant to provide a "high-resolution snapshot" of marine chemical ecology at the beginning of the 21st century. Some 38 authors have prepared 18 chapters that range from ecological and evolutionary perspectives to detailed studies of specific interactions. Many of the recognized gurus in the field have contributed with a few conspicuous absences (Faulkner, Fenical, Hay).

The book is in four sections with Chapters 1–3 examining distributions of secondary metabolites in major animal phyla and discussions of marine natural products from both ecological and evolutionary perspectives. The attempt at this broad overview is laudable, and I found the chapters readable and reasonable. Marine chemical ecology has lagged significantly behind terrestrial chemical ecology, and attempts at such syntheses are needed. Chapters 4–10 are organismal in approach and include work on benthic invertebrates, meroplankton and holoplankton, algal/herbivore interactions, antarctic organisms, spatial patterns of algal chemical defense, resource allocation, and surface colonization. While detailed in nature, they are engaging enough for the terrestrial chemical ecologist to follow and wonder about.

The third section, Chapters 11–15, focuses on cellular and physiological aspects. Physiological consequences of natural products ingestion, exploitation of chemical gradients by algae and bacteria, chemical cues for larval settling and metamorphosis, prey attraction, chemoreception, and UV absorbing compounds are the topics considered. The work of major researchers in the fields is represented in these cafeteria-style offerings. The final three chapters venture into potential practical applications of marine chemical ecology. The two well-known areas of biomedical prospecting and antifoulant development provide the central thrust here. The final chapter focuses on the enormous diversity of known compounds from microorganisms and the need to focus on new methods for their isolation and cultivation.

While this book is certainly no "Harborne" (Introduction of Ecological Biochemistry, now in its 4th edition), in that it is neither concise, nor focused on the

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major phenomena operating in marine (as opposed to terrestrial systems), it does fill a major void. The science is high caliber, the chapters are eminently readable and well-edited, a lot of excellent science will be prompted by what is included, and there is something for the tastes of nearly everyone. One does get, as the book jacket claims, "an integrated perspective of the current state of the this discipline, and its future. It is worth the money and needs to be on the book shelves of those working in this exciting and growing discipline. It could also probably be used successfully as a text for graduate courses.

John T. Romeo
Department of Biology
University of South Florida
Tampa, Florida 33620

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HOT EXTRACTION AND CHARACTERIZATION OF A LIGNINLIKE FRACTION INVOLVED IN LARVICIDAL EFFECTS OF DECOMPOSED LEAF LITTER AGAINST MOSQUITO

MATHIEU TILQUIN,[1] JEAN-CLAUDE MEYRAN,[1,*] MARCEL ASTHER,[2] and GERARD MARIGO[1]

[1]*Laboratoire "Ecosystèmes et Changements Environnementaux"*
Centre de Biologie Alpine, Université Joseph Fourier
BP 53, 38041 Grenoble Cedex 9, France

[2]*INRA, Unité de Biotechnologie des Champignons Filamenteux*
IFR-BAIM, ESIL. CP 925
Faculté des Sciences de Luminy
13288 Marseille Cedex 09, France

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Abstract—Hot water-extraction was performed on decomposed leaf litter in order to solubilize the toxic fraction involved in the dietary interaction against mosquito larvae in subalpine breeding sites. The toxic fraction was partially extracted by water with an optimum temperature of 60°C and recovered in an insoluble form. Phytochemical characterization was achieved through differential enzymatic hydrolyses, using the laccase mediator delignifying system, and aluminum chloride chelation monitored by standard bioassays; comparative spectrophotometric analyses in ultraviolet light after solubilization in acetyl bromide; and comparative reversed-phase high-performance liquid chromatography of the phenolic aldehydes after alkaline nitrobenzene oxidation. The results suggested the involvement of ligninlike compounds in the toxicity of the isolated fraction. Toxicity of this fraction appeared far stronger than that of the crude leaf litter. The involvement of this ligninlike fraction in the dietary toxicity of leaf litter against larval mosquito was then investigated.

Key Words—Leaf litter, larvicidal activity, ligninlike compounds, hot-water extraction, *Aedes aegypti*.

* To whom correspondence should be addressed. E-mail: jcmeyran@ujf-grenoble.fr

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INTRODUCTION

Plant–insect dietary interactions are often mediated by polyphenols that are among the most ubiquitous vegetable xenobiotics (review in Bennett and Wallsgrove, 1994). These plant secondary compounds act as chemical barriers in ecological and evolutionary patterns of terrestrial herbivorous insects (review in Ayres et al., 1997) and aquatic detritivorous Arthropoda (Newman, 1991).

Recently, foliar polyphenols were found to be involved in a particular dietary interaction between the detritivorous aquatic arthropodan fauna and the leaf litter of the arborescent vegetation decomposing in the water of the subalpine mosquito breeding sites (Rey et al., 1996). Owing to their differential biocidal effects against larval Diptera (Rey et al., 1998a; David et al., 2000a) and planktonic Crustacea (Pautou et al., 2001), leaf litter polyphenols are involved in niche differentiation of these aquatic populations and are considered as bioindicators (Rey et al., 2000). Such a chemical interaction was investigated by using either tannic acid (a commercially available standard polyphenolic mixture) (Nishizawa et al., 1982) or crude decomposed alder leaf litter originating from the mosquito breeding sites. The toxicity of those polyphenols is exerted against the midgut epithelium of consumers (Rey et al., 1999; David et al., 2000a), thus leading to pathological effects stronger than those obtained after treatment with the bacterioinsecticide *Bacillus thuringiensis* (Rey et al., 1998b). Because the biocidal properties of crude alder leaf litter are also stronger than those of tannic acid, the possible use of this vegetable leaf detritus was suggested for integrated larval mosquito control (Rey et al., 2001).

Preliminary ecotoxicological analyses have indicated that dietary toxicity was undetectable in green leaves and that the settling of the leaf litter toxicity required a 10-month long decaying process in the water of the mosquito breeding site, under complex seasonal environmental factors (David et al., 2000b). However, the possible generation pattern of the toxic molecules (i.e., de novo generation or strip mining from a complex preexisting matrix) during the decaying process was still unknown.

Fractionation procedures at room temperature using water, organic solvents, and a detergent allowed us to isolate an insoluble toxic residue corresponding to a cell-wall fraction associated with high phenolic activity (David et al., 2000b). Additional enzymatic and alkaline/acid hydrolyses did not affect either toxicity or phenolic activity of the cell-wall fraction, contrary to diverse chemical oxidations, suggesting the possible involvement of ligninlike compounds in the toxicity (David et al., 2001). During these characterization steps, laboratory treatments monitored by a standard larval mosquito bioassay have indicated that the toxicity of the decomposed leaf litter was heat-sensitive (David et al., 2000b), but the origin of this heat sensitivity (i.e., through extraction or breakdown of the toxic molecules) remained unknown.

In this work, we considered a possible solubilization of the toxic fraction using hot water. Once extracted, the following objectives were explored: (1) determine the relative stability of the aqueous extract and the evolution of its larvicidal properties under controlled conditions; (2) check the ligninlike hypothesis of David et al. (2001); (3) evaluate the relative toxicity of this fraction to that of the crude leaf litter, in order to check the efficiency of the extraction procedure and the real larvicidal potentialities of the toxic molecules.

METHODS AND MATERIALS

Plant and Insect Material. Extraction and characterization of the toxic fraction was investigated on 10-month decomposed poplar leaf litter samples known for their acute toxicity toward larval mosquitoes (David et al., 2000b) and collected in a woodland submitted to periodic flooding in the Rhône-Alpes Region of France (Pautou and Girel, 2001). Experimentation was carried out either on crude leaf litter or on a standard lignin fraction purified from crude leaf litter according to the procedure of Alibert et al. (1969). All fractions were air-dried and preserved deep frozen until use.

Toxicity controls of all crude material and extracted or treated fractions were performed on *Aedes aegypti* L. fourth-instar larvae (Bora-Bora strain) raised in our laboratory (Rey et al., 1999) and used as a standard for bioassays.

Hot Aqueous Extraction of Toxic Fraction. Extraction of the toxic fraction was performed on 150 mg dry crude leaf litter maintained under gentle stirring in 30 ml distilled water for 1 hr at constant temperature. Temperatures assayed varied from 20° to 100°C. The remaining solid residue was discarded after separation from the aqueous extract by centrifugation (5000 rpm for 5 min), then filtered through one layer of Whatman No. 4 filter paper (pore size 25 μm).

Toxicity and Stability Checking of Aqueous Extract. Toxicity of the aqueous extract was checked using the standard *A. aegypti* bioassay design of David et al. (2000a,b, 2001). The assay was conducted at 25°C on homogenous samples including 20 same-sized larvae, in glass vials containing 10 ml of aqueous extract buffered to pH 7.5. Larval mortality was measured after 4 hr of exposure and the results corrected by Abbott's formula (Abbott, 1925). In order to investigate the relative importance of the concentration (C) versus dose (q) of the aqueous extract in the larval toxic response, the initial extract was diluted from C to C/2 and C/4, and the bioassay volume was increased from 5 to 10 and 20 ml, thus supplying the same dose to the larvae. Then the bioassay volumes of the C/4 solution were reduced from 20 to 10 and 5 ml, thus dropping the dose available for the larvae from q to q/2 and q/4.

The persistence of toxicity in the aqueous extract was monitored by successive bioassays performed daily during a week on 10-ml aliquots. At the same time, stability of the aqueous extract was checked by visual observation.

Separation and Phytochemical Characterization of an Insoluble Fraction in the Aqueous Extract. A dense fraction was progressively observed within the aqueous extract, which separated little by little with time from the solution as it was cooling down and until the extract was totally cloudy. This insoluble fraction (IF) was isolated from 24-hr-old aqueous extracts either by centrifugation (5000 rpm for 30 min) or by filtration through a 0.45-μm filter (Millipore, type HV). To check toxicity, the supernatant and filtrate obtained with 10 ml aqueous extract (corresponding to about 50 mg dry crude leaf litter) were used directly for bioassays, whereas the filtered IF and pellet were resuspended in 10 ml water before being bioassayed.

In order to check the involvement of ligninlike molecules in the toxicity according to the hypothesis of David et al. (2001), the IF was submitted to several characterization tests: differential enzymatic degradation and chelation with aluminum chloride ($AlCl_3$) (Jurd, 1962) monitored by toxicity controls; spectrophotometric analysis in ultraviolet light after solubilization in acetyl bromide (Johnson et al., 1961), which appears as the most feasible, specific, and reproducible direct method for lignin characterization (Alibert and Boudet, 1979; Fukushima and Dehority, 2000); and reversed-phase high-performance liquid chromatography (HPLC) analysis of the phenolic aldehydes after alkaline nitrobenzene oxidation (Hartley, 1970). This chemical degradative procedure is frequently used for lignin characterization because it provides, in satisfactory yield, the three major monomeric units of lignins (review in Lapierre et al., 1995). HPLC is a routine technique for qualitative and quantitative characterization of these phenolic aldehydes (Villeneuve et al., 1982).

Chelation with 0.05% $AlCl_3$was performed at 20°C and pH 7 for 1 hr on the IF recovered from 10 ml of aqueous extract. Enzymatic degradation of the IF was performed according to the procedure of David et al. (2001), using an enzymatic mix made with 10 mg caylase (caylase 345, Cayla S.A., Toulouse, France) and 1 mg pectolyase (Sigma), to solubilize the polysaccharide and pectin compounds, 1 mg β-glycosidase (2.5 units/mg, Sigma) and 5 μl esterase (4.16 units/ml, Sigma), to break down glycosidic and ester linkages, respectively. The IF recovered from 10 ml aqueous extract was treated with 1 ml enzymatic mix for 60 min at 30°C. After enzymatic treatment, the supernatant was removed by centrifugation (10,000 rpm for 5 min). The insoluble residue was water-rinsed and centrifuged (10,000 rpm for 5 min) before resuspension in 10 ml water for standard bioassay.

Laccase digestion was performed through a laccase-mediator delignification system using *N*-hydroxybenzotriazole (HBT) as a chemical mediator. Highly purified laccase from the white rot fungus *Pycnoporus cinnabarinus* and HBT were from M. Asther. The IF recovered from 10 ml aqueous extract was resuspended in 1 ml HBT solution (0.1%) buffered to pH 5 with 0.05 M sodium tartrate/tartric acid. The reaction was initiated by dissolving 30 units of solid laccase in the reaction medium and digestion was conducted for 6 hr at 30°C with strong agitation

every hour for regular oxygen supply. The supernatant was then removed by centrifugation (10,000 rpm for 5 min). The residual pellet was rinsed in water, then resuspended in 10 ml water before standard bioassay.

Before comparative spectrophotometric analysis using an Uvikon 933 spectrophotometer, samples of standard lignin fractions and of hot-water-extracted IF were treated for 30 min at 70°C with 25% acetyl bromide in acetic acid. Excess reagent was removed by adding 7.5 M hydroxylamine hydrochloride. Samples were diluted with a blank solution to give an absorbance between 1.5 and 2.5.

Phenolic aldehydes were obtained from 10 mg standard lignin fractions and 5 mg IF after alkaline nitrobenzene oxidation following the procedure of Hartley (1970). The aldehyde fraction was purified by successive extractions with diethyl ether, first at pH 12, in order to eliminate nitrobenzene, and then at pH 8.5, in order to extract the phenolic aldehydes. For HPLC analysis, a reversed-phase system, including a Waters Spherisorb 5 μm ODS-2 column (250 $\times$ 4.6 mm ID, Alltech, Deerfield, Illinois, USA) was used. The column was equipped with a 5-mm precolumn, a Dionex 4500i gradient pump, Shimadzu SIL-9A (autoinjector), Shimadzu SPD 6A (UV detector), and Shimadzu Workstation Class CR-10 (integration software). The mobil phase consisted of ultrapure water with 0.01% orthophosphoric acid and methanol (HPLC grade). The column was eluted with a 30-min isocratic 70:30 water–methanol solution at a flow rate of 1 ml/min. Samples of 20 μl were injected in the column and phenolic aldehydes were detected at 280 nm.

Toxic Efficiency of the Insoluble Fraction. In order to determine the toxicity–dose relation and, thus, the toxic efficiency of the hot-water-extracted IF, the amount of organic material fed to larvae in bioassays was accurately estimated by the determination of carbon in IF samples using a CHONS Microanalyser (NA 1 500 from Carlo Erba Strumentazione).

RESULTS

Optimal Conditions for Hot Aqueous Extraction of the Toxic Fraction. As shown in Figure 1, toxicological comparison of the aqueous extracts obtained at different temperatures revealed a gradual increase in toxicity when the extraction was performed between 20° and 60°C, in parallel with a gradual decrease in the toxicity of the residues. When extraction was performed between 60° and 100°C, toxicity progressively decreased in the aqueous extracts, and no remaining toxicity was observed in the residues. Thus, 60°C was the optimal temperature for an efficient water extraction of the toxic molecules from decomposed leaf litter.

Instability of Aqueous Extract and Toxicity of Insoluble Fraction. For five days after the extraction process, aqueous extracts stored at either 4°C or 20°C always gave 100% larval mortality (data not shown), thus indicating that storage

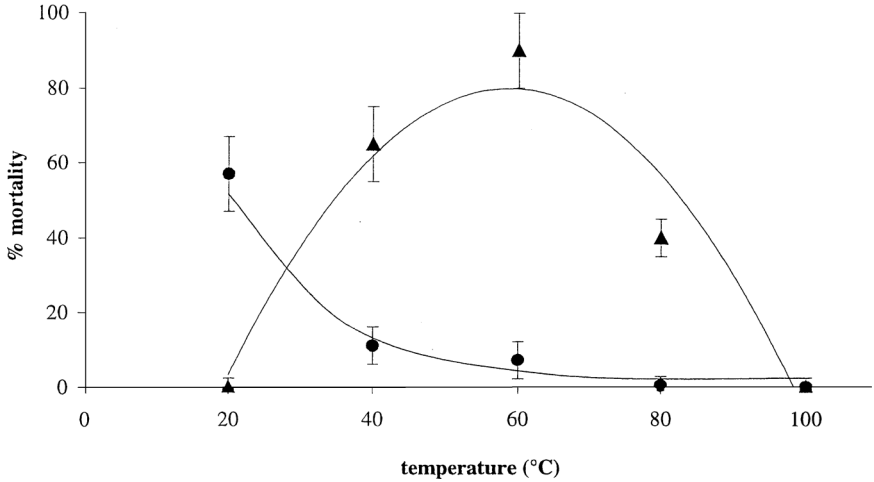


FIG. 1. Effect of temperature on the aqueous extraction of the toxic fraction from crude leaf litter (▲, toxicity of the aqueous extract; ●, toxicity of the residue; % mortality ± SE calculated from triplicate runs).

conditions did not affect the extract toxicity. However, as early as the first day of storage, the initial clear solution became cloudy, and an insoluble phase appeared and gradually increased. The progressive precipitation of an insoluble phase in the medium could explain the relative increase of the aqueous extract toxicity [i.e., from 80% to 100% larval mortality after a five-day storage (data not shown)]. Such an increase in toxicity may be related to the bioavailability increase of this toxic fraction for larval ingestion. The importance of bioavailability of the toxic molecules in the bioassay medium was clearly indicated by comparison of the effect of different concentrations of this toxic fraction for a given dose (Figure 2a) versus the effect of different doses for a given concentration (Figure 2b). This comparison showed that larval response depends on the overall dose of toxic fraction available in the bioassay solution, rather than on its concentration. Moreover, the larvicidal effect was lost in the filtrate or supernatant of filtered or centrifuged aqueous extracts, in contrarst to the whole extract or pellet, in which the whole toxicity was recovered (data not shown). All these results are in agreement to suggest a larval bioavailability of the toxic molecules in the aqueous medium in an insoluble form.

Characterization of the Toxic Insoluble Fraction. Enzymatic digestion of the toxic IF with the enzyme mix of David et al. (2001) did not alter the larvicidal effect (Table 1). In contrast, the toxicity of this fraction was entirely lost after the action of the laccase-mediator delignification system, whereas laccase and HBT alone had no larvicidal effect. These results suggest that lignins are good candidates for the toxicity. Moreover, since toxicity was not observable after treatment with $AlCl_3$,

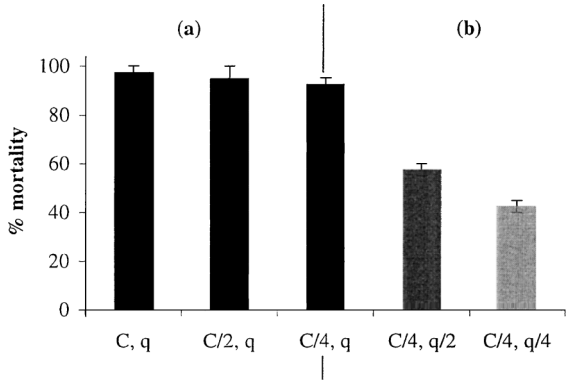


FIG. 2. Larvicidal effect (% mortality ± SE calculated from triplicate runs) of aqueous extract depending on (a) the concentration (ranging from C to C/4) and (b) the dose available (ranging from q to q/4 in 10 ml of bioassay medium.

which is a well-known hydroxyphenolic complexing agent, it may be concluded that OH phenolic groups are specifically involved in the larvicidal effect.

The UV spectra obtained with the standard lignin fraction and the toxic IF appeared to be similar, particularly between 270 and 290 nm (Figure 3). This suggests that part of the lignin constituents of the decomposed leaf litter were extracted by our hot-water procedure.

The presence of ligninlike molecules in the IF was also confirmed by HPLC profiles of the aldehydes after alkaline nitrobenzene oxidation (Figure 4). However, comparison of the respective profiles of standard lignin fractions and toxic IF (Figure 4) showed some differences in the aldehyde composition with maintenance of the relative proportion of *p*-hydroxybenzaldehyde (18 vs. 12 μmol/g, respectively in the standard lignin fraction and in the IF) and disappearance of vanillin and syringaldehyde from the IF.

TABLE 1. EFFECTS OF ENZYMATIC AND $AlCl_3$ TREATMENTS ON TOXICITY OF INSOLUBLE FRACTION

	Toxicity (% mortality)		
Control	Enzymatic mix[a]	Laccase[b]	$AlCl_3$
100	100	0	0

[a] The enzymatic mix was made of caylase, pectolyase, β-glycosidase and esterase.

[b] The laccase was used in the presence of the chemical mediator HBT. Each trial was repeated three times, SE < 5% is not shown.

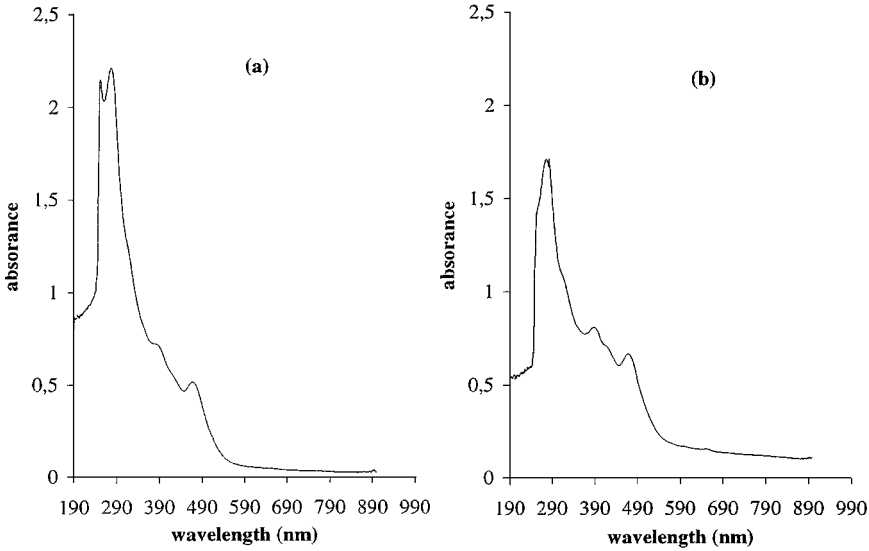


FIG. 3. Comparative spectrophotometric profiles of (a) standard lignin fraction and (b) toxic insoluble fraction after solubilization in acetylbromide 25% in acetic acid. The samples were diluted with acetic acid to give an absorbance between 1.5 and 2.5.

Larvicidal Activity of the Insoluble Fraction. Comparison of the toxic effects of different amounts of IF in the bioassay medium (Figure 5) revealed a larvicidal effect detectable using amounts as low as 18 μg. This toxicological effect increased sharply between 40 and 70 μg to reach a maximum at nearly 80 μg. Under such experimental conditions, an LD_{50} (dose generating 50% mortality) of almost 50 μg was observed, thus indicating the very strong toxic power of the IF.

DISCUSSION

The toxic fraction of decomposed leaf litter involved in the dietary interaction against larval mosquitoes in subalpine breeding sites was previously assessed as a heat-sensitive, insoluble, phenolic, cell-wall fraction indirectly recognized as associated with ligninlike compounds (David et al., 2000b, 2001). The current phytotoxicity-based, gentle, hot-water extraction procedure allowed us to purify, with an acceptable efficiency, an unstable fraction showing strong toxicity. The intrinsic water insolubility, together with some phytochemical characteristics of this fraction, allowed us to recognize it as a true ligninlike fraction, and its involvement in the dietary toxicity of the decomposed leaf litter in subalpine mosquito breeding sites may be questionned.

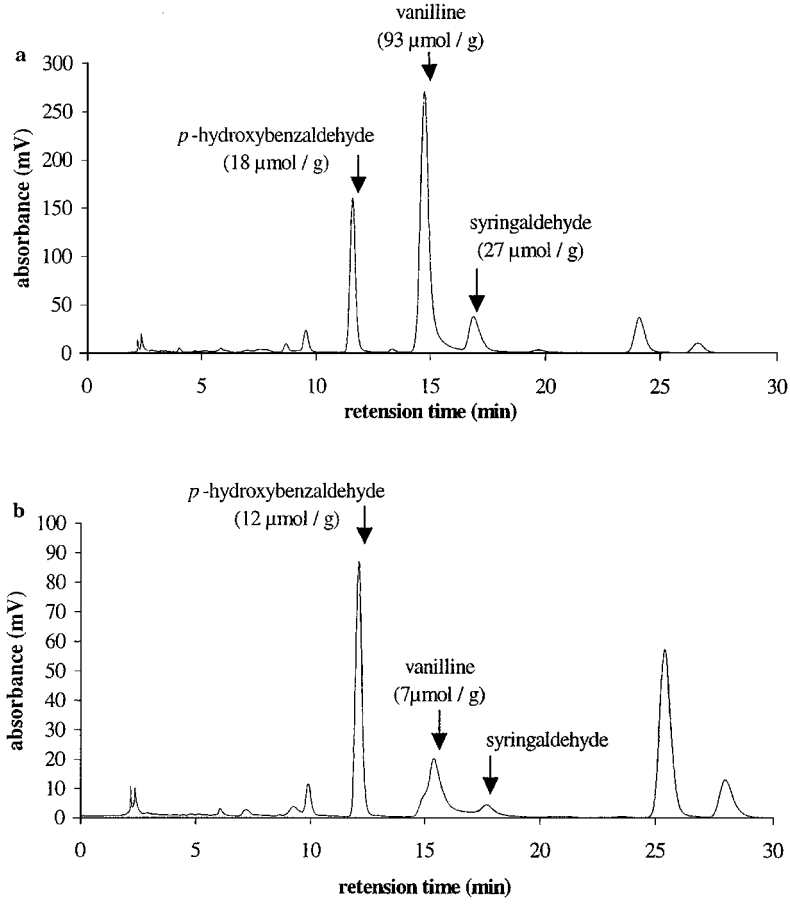


FIG. 4. Comparative HPLC profiles of standard lignin fraction (a) and toxic insoluble fraction (b) after alkaline nitrobenzene oxidation. Peaks identified are indicated on the chromatogram. The amount of each aldehyde (per gram of dry material) is indicated in parentheses.

Characterization of Ligninlike Compounds in the Insoluble Toxic Fraction. Whereas the preliminary characterization of ligninlike compounds in the larvicidal fraction of the decomposed leaf litter by David et al. (2001) was indirect, because it was performed on a nonextracted insoluble cell-wall fraction, the current analysis involves a temporarily solubilizable fraction that allowed the concentration of toxic molecules. The efficiency of our hot-water extraction procedure is evident when the toxicity of extracted IF ($LD_{50} = 50\mu g/10$ ml) is compared to that of the cell-wall fraction ($LD_{50} = 4$ mg/10 ml) found by David et al. (2000a).

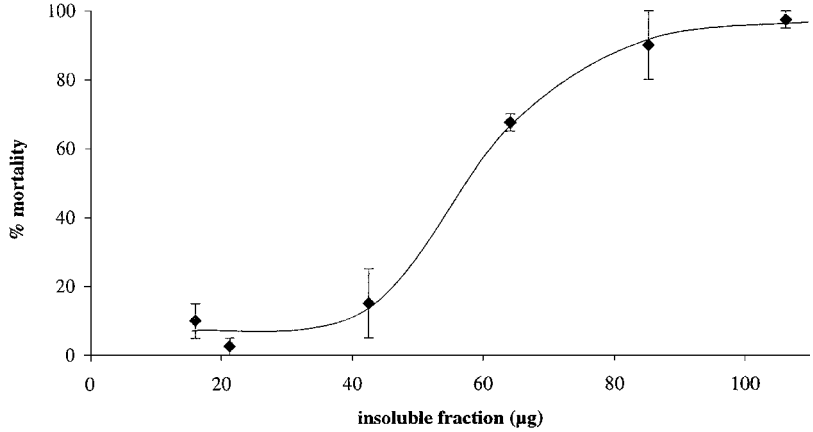


FIG. 5. Dose–response of *A. aegypti* larvae (% mortality ± SE calculated from triplicate runs) to the toxic effect of the IF. The amount of insoluble fraction fed to the larvae in the 10-ml bioassay was estimated by the determination of carbon.

Insolubility of the toxic leaf-litter molecules at ambient temperature in both water and organic solvents (not shown) is the first characteristic in favor of a ligninlike hypothesis. This water-insolubility is accompanied by a lack of toxicity in the water leached from immersed leaf litter during a 10-month decaying process in subalpine mosquito breeding sites (David et al., 2000b). In the same way, preliminary data correlate the toxicity of decomposed leaf litter to a water-insoluble phenolic fraction that is bound to the cell wall (David et al., 2000b). Solubilization of these toxic molecules in water has necessitated concomitant heat treatment, similar to some lignin forms (Lai and Sarkanen, 1971). Their instability at high temperature, which has also been reported through previous analysis of the cell-wall fraction (David et al., 2001), suggests the particular features of these ligninlike compounds. However, whereas these toxic molecules can be optimally extracted at 60°C, they progressively precipitate after this extraction step, thus finally revealing a water-insolubility typical of lignin compounds.

This toxic insoluble fraction is difficult to characterize directly, as generally observed for lignins (Higuchi, 1971), thus requiring the use of degradative reactions. The reactions used in the present study are typically employed in the characterization and structural exploration of lignin molecules (review in Lapierre et al., 1995).

The occurrence of resistant bonds in these biopolymers is responsible for the poor yields of most of the enzymatic degradative procedures performed on lignins. However, laccases from white-rot fungi [e.g., *Pycnoporus cinnabarinus*, which produces high levels of laccase (Sigoillot et al., 1999; Cameron et al., 2000; Herpoël et al., 2000)] are one of the best natural lignin degrading agents

(Reid, 1995; Herpoël et al., 2001). Their efficient in vitro use necessitates the presence of a mediator that is oxidized by the enzyme. This activated mediator then oxidizes the lignin molecules, which subsequently fragment (Sealey et al., 1998). The suppression of toxicity in the IF treated by laccase is strongly suggestive of the ligninlike nature of the larvicidal molecules in the decomposed leaf litter. A ligninlike character is also suggested by our inability to cancel the toxicity of the IF treated with the enzymatic mix of David et al. (2001), which solubilizes nonlignin cell-wall components and glycosidic and ester linkages (Wallace and Fry, 1994). Insensitivity to $AlCl_3$ treatment is further indirect evidence of the involvement of ligninlike molecules. $AlCl_3$ is known to complex phenolic hydroxyl groups (Jurd, 1962) in plant polyphenols in general, and lignins in particular.

More direct evidence that demonstrates the involvement of ligninlike molecules in this larvicidal effect was found by comparing the UV absorption spectra of the standard lignin fraction to that of the IF after treatment with acetyl bromide to solubilize lignin (Johnson et al., 1961). The similarity between these two UV spectra suggests that lignin compounds were extracted from decomposed leaf litter by hot water. The presence of lignin in the IF was also confirmed by thioacidolysis (data not shown), another method used to characterize lignin (Lapierre et al., 1986). Comparison of the monomeric units obtained after nitrobenzene oxidation suggests that hot water enhances the preferential solubilization of some forms of lignins—the *p*-hydroxybenzaldehyde units in particular (arising from the coumarylic alcohol) that differ from the native lignins in which three monomeric units (i.e., *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde) were present.

Possible Involvement of Ligninlike Compounds in the Larvicidal Effect of Decomposed Leaf litter. The involvement of a ligninlike fraction in the dietary toxicity of decomposed leaf litter against larval mosquitoes is plausible. Lignins constitute the most stable and dominant components in decomposed vegetable material because of their resistance to most of the biotic and abiotic processes involved in leaf litter processing (Criquet et al., 2000). Moreover, lignins are known to have deleterious effects against phytophagous consumers—indirectly, through decreasing the digestibility of plant cell-walls, and directly through their own biocidal properties [e.g., against some larval Coleoptera (review in Wainhouse et al., 1990)]. However, characterization of these toxic, ligninlike molecules is still poorly documented. This is not surprising, given the tremendous variety of lignin compounds existing in nature and the difficulties in characterizing them. For example, several lignans with insecticidal properties against larval Lepidoptera have been isolated from herbaceous perennial plants (see references in Taniguchi et al., 1989).

Although further phytochemical investigation is necessary to establish the characteristics of ligninlike compounds that may be involved in leaf litter toxicity against larval mosquitoes, ecological considerations of the pattern of generation of

this toxicity during the leaf litter decaying process may give some preliminary information on these molecules. Contrary to what is generally observed in toxic leaf detritus, where dietary toxicity persists from the green leaf (Valiela et al., 1979), the larvicidal effect of leaf litter gradually appears during a 10-month long decaying process in a given water environment. This pattern of toxicity, previously related to the gradual increase of an insoluble phenolic fraction within the decomposing leaf litter (David et al., 2000b), is currently related to the ligninlike fraction. However, the evolutionary pattern of this fraction during the decomposing process may be either quantitative, through gradual enrichment of preexisting lignins, or qualitative, through alteration of the monomeric composition and possible maturation of these lignins into more condensed forms.

The toxicological interest of ligninlike compounds as larvicidal molecules in the aquatic subalpine mosquito breeding sites comes from their strong water-stability together with their variability in the oxidation state. As they are known to be the most recalcitrant components of decomposed leaf litter, lignins may behave as relatively stable molecules in the aquatic medium together with a bioavailability for detritivorous consumers that is better than toxic water-soluble polyphenols. Such a strong and durable toxicologic yield has been shown by comparison of the larvicidal effects of crude decomposed alder leaf litter versus tannic acid in the aquatic medium (David et al., 2000b; Rey et al., 2001). The involvement of oxidative processes in the leaf-litter larvicidal effects against mosquito has been previously suggested by David et al. (2001), as generally observed in insects fed phenolic-containing vegetable (review in Johnson and Barbehenn, 2000). Autooxidation of phenolic groups may result in the formation of superoxide radicals and other reactive species (Appel, 1993), leading to a direct oxidative stress of the larval digestive system. This is supported in our experiments using the laccase mediator delignifying system, which prevents the larvicidal effect of IF. Indeed, ^{13}C NMR analysis of the structure of residual lignins before and after laccase mediator treatment in kraft pulps (Sealey and Ragauskas, 1998) have shown that, among other modifications, this system selectively oxidizes free phenolic groups in lignins. Since our hot-water extracted ligninlike molecules lose their toxicity after action of the laccase mediator delignifying system, their toxicity may be associated with oxidative processes occuring in the larval gut (Barbehenn and Martin, 1994). Characterization of the chemical modifications induced by the laccase mediator system would be indicative of the lignin fraction or lignin substituents involved in the toxicity. Further analyses of such processes are now in progress in our laboratory.

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PERAMINE ALKALOID VARIATION IN *Neotyphodium*-INFECTED ARIZONA FESCUE: EFFECTS OF ENDOPHYTE AND HOST GENOTYPE AND ENVIRONMENT

STANLEY H. FAETH,[1,*] LOWELL P. BUSH,[2] and T. J. SULLIVAN[1]

[1]*Department of Biology*
PO Box 871501
Arizona State University
Tempe, Arizona 85287-1501, USA

[2]*Department of Agronomy*
University of Kentucky
Lexington, Kentucky 40546-0091, USA

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Abstract—We determined concentrations of peramine, the only alkaloid produced by *Neotyphodium*-infected (E+) Arizona fescue plants (of the four major types typically assayed in infected grasses), in a long-term field experiment. Four plant genotypes with (E+) and without (experimentally removed, E−) their respective haplotypes (two haplotypes in two plant genotypes) of *Neotyphodium* were grown in the field under manipulated soil moisture and nutrients. Peramine production required the presence of the endophyte; plants without their endophytes did not contain peramine. Whereas the endophyte was necessary for peramine production, levels of peramine did not vary by *Neotyphodium* haplotype within plant genotypes. Furthermore, peramine levels did not differ among soil moisture and nutrient treatments, and growth and reproductive measures of the host grass explained little of variation in peramine levels. Instead, peramine levels differed significantly between plant genotypes harboring the same endophyte haplotype, suggesting that plant genotype, rather than endophyte haplotype or environment, largely determines levels of peramine in Arizona fescue. The results suggest that whereas the endophyte is required for peramine production, the plant genotypic background in which the endophyte is embedded, rather than endophyte haplotype or environmental factors, mostly influences peramine levels within this population of Arizona fescue.

Key Words—Alkaloids, Arizona fescue, endophyte, environment, host, genotype, native grasses, mutualism, *Neotyphodium*, peramine.

*To whom correspondence should be addressed. E-mail: s.faeth@asu.edu

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INTRODUCTION

Symptomless, systemic, and asexual fungi in cool season grasses are generally viewed as plant mutualists because they often increase the resistance of their hosts to herbivores, root-feeders, and pathogens and increase competitive abilities (e.g., Clay, 1988, 1990; Saikkonen et al., 1998). Because the asexual endophyte is dependent on the host grass for dissemination to offspring plants, increases in host fitness should result in commensurate increases in endophyte fitness (e.g., Wilkinson and Schardl, 1997; Schardl and Clay, 1997). The primary mechanism of increased resistance to natural enemies of the host plant, as well as some aspects of increased competitive abilities, such as allelopathy (Peters and Zam, 1981; Matthews and Clay, 2001), is fungal-related alkaloids (e.g., Siegel and Bush, 1996; Bush et al., 1997; Schardl and Clay, 1997). The asexual, systemic endophyte in the genus *Neotyphodium*, as well as its closely related sexual form, *Epichloë*, may produce varying levels of alkaloids within four major groups—pyrrolizidine alkaloids (lolines), peramine, ergot alkaloids and indolediterpenoids (lolitrem) (Porter, 1994; Siegel and Bush, 1996; Bush et al., 1997). Whereas fungal alkaloids are the primary route for endophyte-related benefits to the host grasses, they may also represent a source of significant costs (Siegel and Bush, 1996; Faeth and Bultman, 2002; Faeth and Fagan, 2002). Alkaloids are nitrogen-rich compounds. Thus, fungal production of these compounds may compete with host growth and reproduction, especially when resources such as nitrogen are limiting, although nutrient demand for alkaloid synthesis may be partially offset if systemic endophytes increase nutrient uptake by roots (e.g., Malinowski and Belesky, 1999). Furthermore, alkaloids themselves may be toxic to the host (Faeth, 2002; Faeth and Bultman, 2002), as has been shown for constitutively produced alkaloids in plants (e.g., Karban and Baldwin, 1997). Thus, alkaloid production is expected to vary with available resources. In E+ domesticated grasses, alkaloid levels sometimes vary with soil moisture and nutrients (Arechavaleta et al., 1989; Agee and Hill, 1994; Roylance et al., 1994; Malinowski et al., 1998) and temperature (Huizing et al., 1991).

Although alkaloid levels may be influenced by environmental factors, the types and levels of alkaloids appear to depend mostly on endophyte species, strain, or genotype (Siegel et al., 1990; Leuchtmann, 1992, 1997; Bush et al., 1993; Christensen et al., 1993; Leuchtmann et al., 2000) and less so on host grass genotype and environment (e.g., Siegel et al., 1990; Leuchtmann et al., 2000). Endophyte species or genotype influences alkaloid production in both infected agronomic (e.g., Siegel et al., 1990; Bush et al.,1993; Christensen et al., 1993) and native grasses (Siegel et al., 1990; Leuchtmann et al., 2000). Endophytic fungal genes for alkaloid production have been identified in some infected domesticated grasses (Wilkinson et al., 2000). However, artificial transfer of endophytes from one host grass species to another suggests that species and genotypic background of the host grass may also influence alkaloid production (Siegel et al., 1990).

Natural variation in alkaloid levels among grass species and within and between populations has been documented for some infected native grasses (e.g., Siegel et al., 1990; Leuchtmann et al., 2000; Jones et al., 2000; Faeth and Fagan, 2002). Variation is often great, ranging from very high and toxic levels of certain alkaloids to none at all (e.g., Leuchtmann et al., 2000; Jones et al., 2000; Faeth, 2002; Faeth and Bultman, 2002). However, the relative contributions of endophyte and plant genotype and local environmental factors to this wide variation in alkaloid levels of E+ native grasses are largely unknown. To our knowledge, there are currently no studies of infected native grasses that simultaneously test host and endophyte genotype and environmental factors.

We determined how endophyte haplotype and plant genotype and environmental factors affect production of peramine in *Neotyphodium*-infected (E+) Arizona fescue as part of a long-term field experiment. In this experiment, two different genotypes of *Neotyphodium* (i.e., haplotypes) were removed from one half of the ramets of infected maternal genotypes, and the infected (E+) and uninfected (E−) clones of each plant genotype were grown under varying soil moisture and nutrient conditions. We predicted that alkaloid amounts should vary mainly by endophyte haplotype and, to a lesser extent, by maternal plant genotype and available resources. In terms of the latter, we predicted that alkaloids should increase in nutrient rich environments because fungal production of these compounds may represent costs to the host plant (e.g., Marks et al., 1991; Faeth and Fagan, 2002).

METHODS AND MATERIALS

Study Plant and Endophyte. Festuca arizonica (Vasey), Arizona fescue, in the subfamily Pooideae, is a dense, perennial bunch grass that reproduces by seed (USDA, 1988) and is native and widespread in the Southwest United States and in northern Mexico (Kearney and Peebles, 1960). Arizona fescue grows in semiarid Ponderosa pine–bunchgrass communities above 2000 m elevation (Kearney and Peebles, 1960), where soils are low in nutrients and seasonal and yearly droughts are common (Schulthess and Faeth, 1998). The asexual, systemic endophyte, *Neotyphodium starrii*, is common in Arizona fescue populations in Arizona, infecting 60–100% of host plants (Lopez et al., 1995; Schulthess and Faeth, 1998). At least three distinct genotypes of *Neotyphodium* have been isolated from Arizona fescue throughout the Southwest (e.g., White and Cole, 1987; An et al., 1992; C. Schardl, personal communication). *Neotyphodium* in *F. arizonica* populations from Arizona most closely resembles *N. starrii* (White and Morgan-Jones, 1987; White et al., 1993), and we use this convention here. *N. starrii* grows intercellularly in aboveground tissues of Arizona fescue, especially in the leaf sheath tissue (White, 1987; Schulthess and Faeth, 1998). *N. starrii*, like all other *Neotyphodium* species and some species of *Epichloë*, is transmitted by growing into culms and then into

developing seeds. Although *N. starrii* will sporulate under restricted conditions in culture (White and Morgan-Jones, 1987), it is vertically transmitted in nature (Faeth and Fagan, 2002), as are other *Neotyphodium* endophytes (e.g., Clay, 1990; Schardl and Clay, 1997). *Neotyphodium* in Arizona fescue produces only the alkaloid peramine (Faeth and Fagan, 2002; N.S. Hill, personal communication; M.R. Siegel, personal communication), one of the four types of alkaloids known and typically assayed from *Neotyphodium*-infected grasses, and at levels varying from zero to 2.3 μg/g in Arizona populations (Faeth and Fagan, 2002).

Neotyphodium-infected Arizona fescue plants were removed in spring 1997 from an isolated population at Merritt Draw, a drainage meadow on the Mogollon Rim (elevation 2500 m), Arizona. Four maternal plants were randomly selected in 1997 from a pool of about 50 E+ plants in the population. Infection was determined initially by a modified tissue print immunoassay (Gwinn et al., 1991) and later confirmed by staining seeds from each plant after each growing season and examining them for the presence of *Neotyphodium* hyphae (Saha et al., 1988; Schulthess and Faeth, 1998). Haploid, asexual *Neotyphodium starrii* is represented by two distinct haplotypes in the Merritt Draw population, based upon four microsatellite DNA loci from 27 sampled plants (Sullivan and Faeth, 2001; T. J. Sullivan, unpublished data). Two of the four plants harbored one haplotype (termed A) and two harbored the other (termed B). Haplotypes of the experimental plants were confirmed by microsatellite DNA analyses of multiple loci (Sullivan and Faeth, 2001). Other populations of *Neotyphodium*-infected Arizona fescue in Arizona can be represented by a single *Neotyphodium* haplotype [e.g., Flagstaff, Arizona population (Sullivan and Faeth, 2001)] or up to six different haplotypes within a single population [e.g., Clint's Well, Arizona population (Sullivan and Faeth, 2001)].

Field Experiment and Sampling. We sampled living leaf tissue for peramine assays from 172 adult Arizona fescue plants as part of a larger experiment designed to test the effects of infection on growth and performance under varying resource environments while controlling for the effects of endophyte haplotype and plant genotype. In brief (details of this experiment are in Faeth and Fagan, 2002), the four E+ maternal plants, two harboring haplotype A and two harboring haplotype B (termed A-1, A-2, and B-1, B-2, respectively) were split into ramets and treated hydroponically with low levels of the fungicide propiconazole to remove the endophyte (M. Marlatt and C. West, personal communication). Fungicide treatment removes *Neotyphodium* from about 50% of ramets (E−); the remaining infected ramets of one genotype (genotype A) served as fungicide-treated, but infected controls (hereafter, termed E + F) such that any spurious effects of the fungicide on alkaloid levels could be tested. Other ramets were treated hydroponically but without fungicide and, thus, remained infected (hereafter E+). After hydroponic treatment, all ramets were planted individually into 16-oz. cups with native soil and continually split and repotted as they grew for about one year in the greenhouse

to provide cloned replicates. Infection status after cloning and growth in the field was confirmed as described above.

Removal of the endophyte strictly controls for plant genotype but does not control for endophyte haplotype. To strictly control for endophyte haplotype, reciprocal inoculations of endophytes from endophyte-removed maternal plants is necessary. While inoculations of uninfected, new seedlings under sterile laboratory conditions are possible for agronomic (e.g., Latch and Christensen, 1985; Christensen, 1995) and a few native grasses (e.g., Meijer and Leuchtmann, 2001), inoculations of adult ramets has thus far only recently been accomplished with the related *Epichloë* endophyte (Johnson-Cicalese, et al. 2000), but not *Neotyphodium*. Inoculation at the seedling stage does not control completely for plant genotype because each seedling is, at best, a full sibling due to the genetic contribution of the male parent. Moreover, inoculations of one haplotype into a maternal plant genotype may result in haplotype-genotype combinations that would not normally occur in nature because the endophyte is strictly maternally transmitted. We note that our experiment using endophyte-removed maternal ramets with known endophyte haplotypes to test how endophyte and plant genotype and varying environments affect alkaloid production is the first, to our knowledge, for a native grass.

We assumed that the maternal plants, two of which harbored haplotype A and two haplotype B, differed genetically from each other. This assumption is reasonable since Arizona fescue forms distinct bunches, outcrosses to produce seed, and does not reproduce by rhizomes (USDA, 1988). All four of the selected maternal plants were at least 10 m from each other. Obviously, plants with different endophyte haplotypes originated from different maternal lines because *Neotyphodium* is maternally transmitted. Maternal plants with the same haplotype are, at worst, half-sibs and probably less related than half-sibship because of outcrossing and very low dispersal distances of seeds from maternal plants ($<$2 m, S. H. Faeth, personal observation).

After growing for one year in the greenhouse, the plants were transplanted into a plot (1 m apart, randomly assigned) at the Arboretum of Flagstaff (elevation 2500 m), where Arizona fescue is the dominant native grass, in June 1998. The arboretum is fenced (4 m high) to exclude livestock and large native grazers (elk and deer) but subjected to herbivory from small vertebrates and invertebrate herbivores and root-feeders. All transplanted plants were approximately the same size ($\sim$10 tillers each). Before planting, the plot was disked to remove native vegetation, which was mostly Arizona fescue and a few other native grasses and forbs. Each plant was randomly assigned one of the following treatments: (1) ambient water and ambient nutrients, (2) supplemental water (1000 ml/plant every three days), (3) added nutrients (1.5 g/liter; 30 N–15 P–30 K fertilizer, every two weeks, and (4) added water (every three days) plus supplemental nutrients (every two weeks). Treatments began immediately after transplanting in 1998 and continued during the next two growing seasons (May–September of 1999 and 2000). All

genotype, infection status, and treatment combinations, including E + F plants, were replicated five times for a total of 180 plants. Eight plants died during the course of the experiment and, thus, 172 plants were sampled for peramine. Ambient precipitation at the arboretum is typically low from April through June each year ($\bar{X} = 16$ mm/month, 50-year average) until summer rains begin in July and August and then again in October and November ($\bar{X} = 38$ mm/month, 50-year average) until winter precipitation begins in December. Soil moisture measurements in the 2000 growing season showed that ambient soil moistures were ~20% saturation from April to June) and increased to 40% saturation in July and August, when summer rains began. Supplemental water treatments increased soil moisture to ~80% saturation throughout the growing season. Likewise, ambient soil nutrients are low (~1–2 ppm N) in native soils at the arboretum (Schulthess and Faeth, 1998), and the level of fertilizer used significantly increases nitrogen content of Arizona fescue plants (Saikkonen et al., 1999). The plot (except for a circular area around each plant to allow aboveground expansion) was covered with a weed barrier (Dalen Co.) that permits water and nutrient penetration but prevents weed growth and hence unwanted plant competition. The barrier was then covered with commercial Ponderosa pine bark chips.

We sampled living leaf tissue from the 172 plants in September 2000 (the end of the growing season but before plants senesced) when plants were harvested in connection with the larger experiment. At this time, growth and reproductive parameters of each plant were also determined. In brief (see Faeth and Fagan, 2002, for details), above- and below-ground mass, mass of seeds, and growth rate were determined. The latter was estimated by dividing final above-ground volume by the above-ground volume at the beginning of growing season. We could not determine any seasonal variation in peramine because the design of the larger experiment did not allow for periodic tissue removal. Tissue samples were placed on dry ice in the field and returned to the laboratory where they were kept frozen and then lyophilized and ground into a fine powder.

Determination of Peramine Levels. All peramine analyses were conducted at the University of Kentucky by LPB. Briefly, peramine was extracted from 150 mg samples and concentration determined by thin-layer chromotography (TLC) modified from Fannin et al. (1990). Then 1200 μl of 80% ethanol was added to the sample in a microcentrifuge tube, the sample was mixed and sonicated, and shaken for 1 hr. After 1 hr, tubes were again mixed, sonicated, and centrifuged. Five hundred microliters of supernatant was removed and taken to dryness in a stream of nitrogen. The residue was dissolved in 50 μl ethanol and 10 μl applied to a reversed-phase TLC plate. The spot was placed about 2.5 cm from one edge of the plate. Development was done in two directions. The edge of the plate opposite the origin was immersed in 0.1% H_3PO_4, pH 7.25, and 60% methanol, and the solvent was allowed to migrate to the top of the plate. The plate was then removed and a strip of adsorbent, 14 mm wide from the edge to the plate towards

the origin, was removed. After drying, the plate was developed in 0.6 M NaCl in 50% methanol removed from the tank, and sprayed with van Urk's reagent. After it was thoroughly dry, the plate was scanned in a Shimadzu High-Speed Thin-layer Chromato-Scanner (model CS-920) at 600 nm in the reflectance mode. The amount of peramine present was determined from a standard calibration curve for each run with authentic peramine. Peramine concentrations are expressed as micrograms per grams of dry mass of the plant.

Statistical Analyses. Peramine levels were not detected in any of the endophyte-removed (E−) plants, even in trace amounts. Therefore, statistical tests for differences between E+ and E− plants were irrelevant. For the E+ plants, we used ANOVA (Systat, 2000; GLM procedures), with plant genotype nested within endophyte haplotype, to test for differences in peramine concentrations based upon haplotype and treatment and their interaction. We also tested for any extraneous effects of the fungicide by comparing peramine levels for E+ and E + F plants (genotype A-1) with a two-factor (fungicide/no fungicide and treatment) ANOVA. All assumptions of ANOVA (e.g., homogeneity of variances) were tested and met. We used Tukey HSD tests to compare differences among levels of factors, when significant.

We used linear multiple regression analyses (Systat, 2000) to determine relationships between peramine content (dependent variable) and growth and reproductive parameters (independent variables). We first used a full multiple regression analysis where all independent variables and the constant were included in the regression model. We then used a backward stepwise regression where each independent variable was removed in a reverse direction from the full model to determine which variables contributed significantly to the regression model.

RESULTS

No peramine was detected (assays were sensitive to 0.003 μg/g) in any of the endophyte-removed plants for any genotype and treatment. Peramine levels did not vary between the E + F fungicide control plants ($\bar{X} = 0.87$ μg/g, SE $= 0.08$) and E+ plants ($\bar{X} = 0.97$ μg/g, SE $= 0.10$) of genotype A ($F = 1.17$, $df = 1,30$ $P = 0.29$), indicating that fungicide treatment did not spuriously alter peramine levels. We expected no differences because the field plants were removed by one year of regrowth, splitting, repotting in the greenhouse, and then >2 years of growth in the field.

For the E+ plants, peramine concentrations did not vary by *Neotyphodium* haplotype (Table 1, no endophyte haplotype effect; haplotype A, $\bar{X} = 0.76$ μg/g SE $= 0.07$; haplotype B, $\bar{X} = 0.82$ μg/g, SE $= 0.08$). Peramine levels did not vary by treatment (Table 1, no treatment effect; Figure 1) nor did haplotype interact significantly with the treatments (Table 1, no treatment haplotype effect).

TABLE 1. ANALYSES OF VARIANCE OF EFFECT OF *Neotyphodium* HAPLOTYPE, PLANT GENOTYPE NESTED WITHIN HAPLOTYPES, AND TREATMENTS ON PERAMINE LEVELS[a]

Source of variation	*df*	Sum of squares	Mean square	*F* ratio	*P*
Endophyte haplotype	1	0.07	0.07	0.46	0.50
Plant genotype within haplotype	2	5.02	2.51	17.51	<0.001
Treatment	3	0.84	0.28	1.96	0.13
Treatment x haplotype	3	0.29	0.10	0.68	0.57
Error	62				

[a] Results are for E+ Plants only because E− (endophyte-removed) had no detectable alkaloids.

Different plant genotypes with the same *Neotyphodium* haplotype varied in peramine levels (Table 1, Figure 2A,B). Plant genotype A-1 had higher peramine levels than did plant genotype A-2 harboring the same haplotype A (Figure 2A). Conversely, plant genotype B-1 had lower peramine levels than did plant genotype B-2 (Figure 2B).

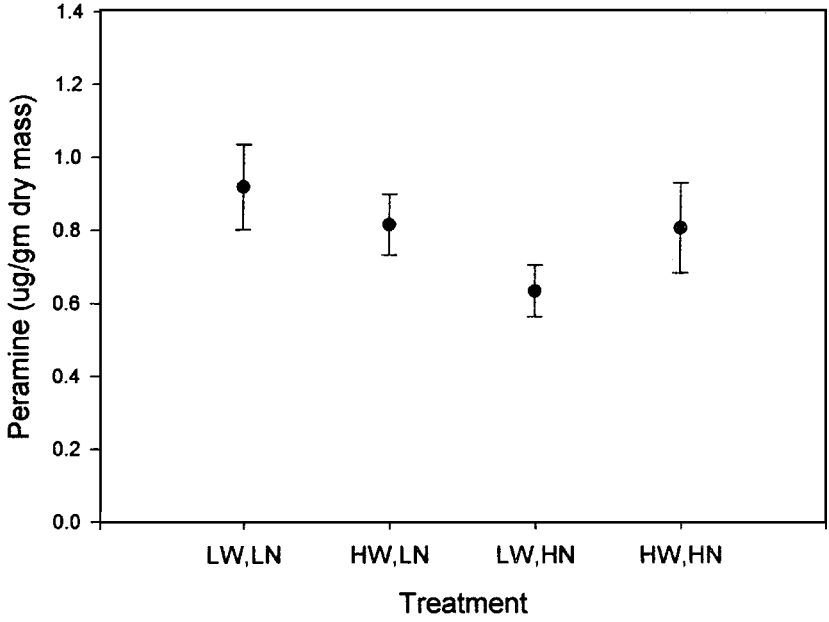


FIG. 1. Peramine concentrations (mean ± SE) of *Neotyphodium*-infected Arizona fescue plants in four water and nutrient treatments (LW,LN = ambient water and nutrients; HW, LN = supplemented water, ambient nutrients; LW,HN = ambient water and supplemented nutrients; HW,HN = supplemented water and nutrients).

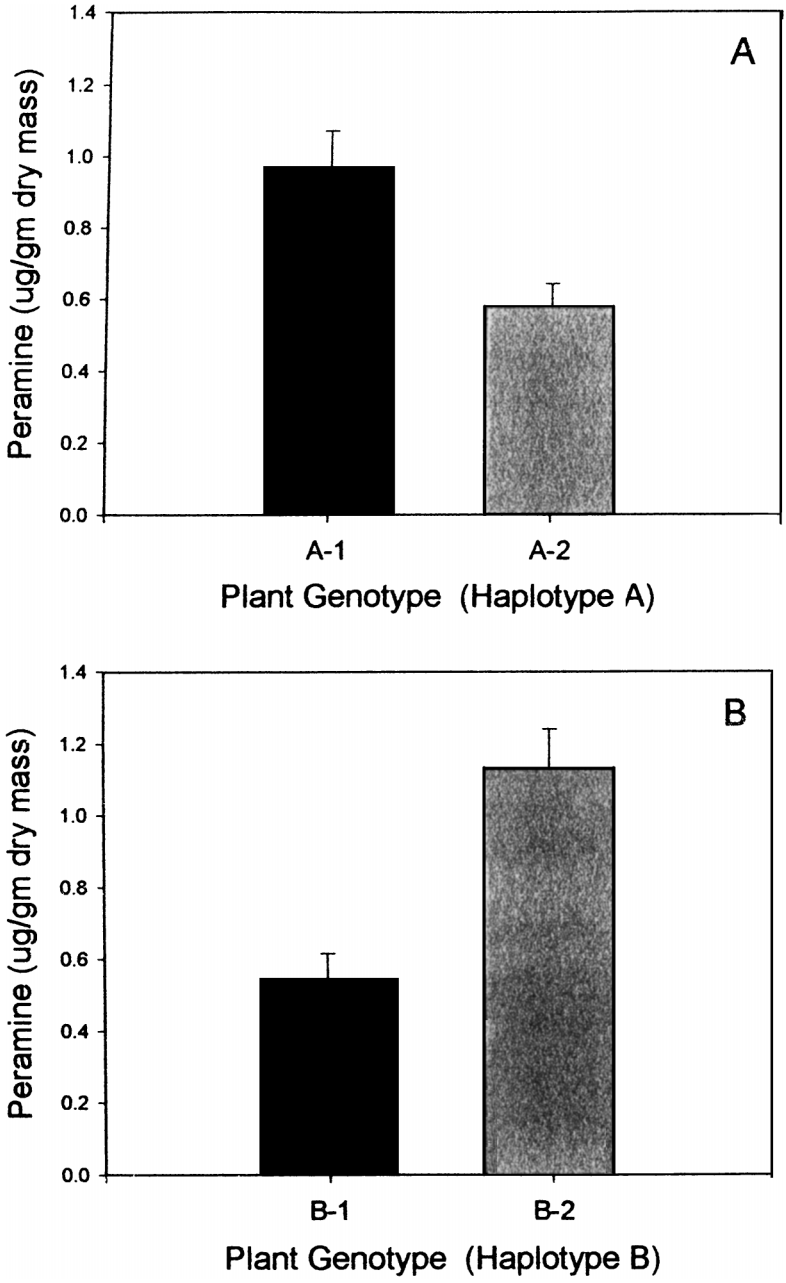


FIG. 2. Peramine concentrations (mean ± SE) of Arizona fescue by genotypes harboring *Neotyphodium* haplotype A and haplotype B.

The full multiple-regression analyses indicated that variation in peramine content was related to host grass growth and reproduction parameters ($F = 2.59$, $df = 5,48$, $P = 0.04$), but the full regression model explained only 13% ($R^2 = 0.131$) of the variation in peramine amounts. Of the five growth and reproductive parameters, only growth rate (as measured by change in above-ground volume from the beginning to the end of the growing season) was significant ($P = 0.003$). Stepwise removal of the other four variables did not affect the overall model. The regression of peramine content with growth rate only showed that peramine levels were positively related to growth rate in the final growing season ($F = 5.10$, $df = 1, 76$, $P = 0.027$), but the regression explained only 6% of the variation in peramine content ($R^2 = 0.06$).

DISCUSSION

The results show that the presence of *Neotyphodium* is necessary for production of the alkaloid peramine in Arizona fescue; plants with their endophyte removed produce no detectable peramine under any environmental conditions. The metabolic origin of alkaloids in endophyte-infected grasses has not always been clear because systemic fungal endophytes in culture may not produce alkaloids, and occasionally alkaloids have been found in host grasses after fungi have been apparently removed (Siegel et al., 1990). Furthermore, some grasses themselves appear capable of producing constitutive alkaloids and other allelochemicals (Chapman, 1996).

Whereas the qualitative production of peramine requires the presence of *Neotyphodium* in Arizona fescue, our results suggest that the host grass genotype, not the endophyte haplotype, determines the quantitative production of alkaloids. Both *Neotyphodium* haplotypes produced different levels of peramine depending on their maternal grass hosts. These results contrast with other studies of alkaloids in E+ grasses where endophyte strain or species is thought to largely dictate alkaloid types and levels (e.g., Siegel et al., 1990; Christensen et al., 1993; Leuchtmann et al., 2000), although many of these cases involved among-host species comparisons. Siegel et al. (1990) found that some endophyte strains in different plant genotypes or species produce different alkaloids, also suggesting that host plant genotype influences alkaloid levels.

Caution is necessary in interpreting the results because we have tested only two *Neotyphodium* haplotypes within two plant genotypes within the same population. Furthermore, identification of endophyte as the same haplotypes via microsatellite DNA, or for the matter, via isozymes, does not necessarily mean those endophytes are genetically identical in peramine-related genes (e.g., Roylance et al., 1994). Including more maternal plant genotypes and endophyte haplotypes from other Arizona fescue populations may complicate the pattern, and additional experiments determining the alkaloid levels of purported haplotypes in

axenic cultures are necessary to confirm our results. We also caution that infected plants could produce other alkaloids besides peramine and be different from the four major groups routinely tested for in endophyte-infected grasses. However, previous experiments and observations on vertebrate (Schulthess and Faeth, 1998; Saikkonen et al., 1999) and invertebrate herbivores (Lopez et al., 1995; Tibbets and Faeth, 1999; Saikkonen et al., 1999; Faeth and Fagan, 2002) suggest this possibility is remote, at least at levels at which herbivores would be negatively affected.

Nevertheless, the results suggest that the widely varying levels of peramine found within the Merritt Draw population of E+ Arizona fescue, and perhaps other populations, may result mostly from the genotypic background of the host. The *Neotyphodium* endophyte is transmitted asexually by hyphae growing into seeds, and, thus, remains genetically uniform within offspring of a maternal plant. Alternatively, genetic recombination during sexual reproduction of the host probably results in many different host genotypes with the same, or very few different, endophyte haplotypes in the host populations. Sullivan and Faeth (2001) found that four populations of Arizona fescue range from a single to six different *Neotyphodium* haplotypes. Multiple haplotypes within the same population apparently result from historical hybridization events between *Neotyphodium* and its sexual ancestor, *Epichloë* (e.g., Schardl et al., 1994; Tsai et al., 1994) or more recent colonization events of E+ seeds or plants with different haplotypes. The latter mechanism, however, seems unlikely given the low dispersal of Arizona fescue. Additional studies are necessary to determine whether these other haplotypes influence peramine levels within and between populations, or if plant genotype remains the dominant factor in determining peramine concentrations.

Our prediction that peramine levels should increase with available resources, particularly nitrogen, was not supported. In agronomic E+ grasses, increased nitrogen (Bacon, 1993; Agee and Hill, 1994), especially under moderate water stress (Belesky et al., 1989; Arechavaleta et al., 1992), may increase alkaloid levels. However, we found no effect of soil moisture or nutrients on peramine production, and no interaction of these abiotic factors with plant genotype (Table 1).

There are several explanations for the lack of effects of soil moisture and nutrient on peramine levels. First, we could not determine seasonal variation because of the design of the experiment, and peramine levels were determined only at the end of the growing season. Whereas levels of peramine at the end of the growing were not affected by the treatments, peramine concentrations may have been related to soil moisture or nutrients earlier in the growing season or in previous years of the study. Seasonal variation in alkaloids occurs for some E+ domesticated (e.g., Belesky et al., 1987; Roylance et al., 1994; Ball et al., 1995; Belesky and Hill, 1997) and native (e.g., Leuchtmann et al., 2000) grasses, although alkaloids levels usually are cumulative seasonally, and the highest levels occur at the end of the growing season (Siegel and Bush, 1996; Leuchtmann et al., 2000). Therefore, we would expect that peramine levels at the end of season should correspond

in relative levels to earlier in the season, even though absolute levels may vary seasonally. Second, we sampled leaf material, which constitutes the bulk of the plants. Alkaloid levels may also vary among plant parts and tissues (e.g., Ball et al., 1997; Belesky and Hill, 1997; Justus et al., 1997; Leuchtmann et al., 2000). It is possible that soil moisture and nutrients may have affected the amounts of peramine in other, nonsampled tissues. However, relative amounts of alkaloids in different plant tissues are usually positively related (e.g., Leuchtmann et al., 2000). A third explanation is that moisture and nutrients were not limiting to plant growth. Thus, peramine production was not altered because host plant stress or nutrient limitation did not occur. However, plants in the supplemented water and nutrient had significantly increased plant growth and reproduction (Faeth and Fagan 2002, Faeth and Sullivan, unpublished). Furthermore, previous experiments using Arizona fescue and the same nutrient manipulations as here, showed increased nitrogen content of the plant (Saikkonen et al., 1999). Therefore, we expected peramine levels, if affected by water stress or nutrient limitation, should have increased in the supplemented nutrient treatments, especially those subjected to low levels of water availability (e.g., Arechavaleta et al., 1992). The results, however, suggest that plant genotype, not environmental factors, explains most of the variation in peramine in E+ Arizona fescue in their semiarid and low-nutrient habitats. Peramine production may be so low, or even nonexistent in some E+ Arizona fescue plants (Faeth and Fagan, 2002), that it would rarely compete with host plant growth and reproduction. If infection increases nutrient uptake by Arizona fescue in their normally poor soils, as demonstrated by Malinowski and Belesky (1999) for E+ tall fescue, then nutrient demand for alkaloid synthesis may also be reduced. This scenario, of course, assumes that alkaloid production is costly to the host, either in terms of production, opportunity, or storage costs (e.g., Bazzaz and Grace, 1997) or autotoxicity to the host (e.g., Karban and Baldwin, 1997). In general, the actual cost of plant defense via allelochemistry is still debated (e.g., Purrington, 2000; Hamilton et al., 2001).

The lack of environmental effects on peramine levels and the low levels of peramine in *Neotyphodium*-infected Arizona fescue found here, as well as in a survey of other E+ Arizona fescue plants in this population (Faeth and Fagan, 2002), suggests that peramine production may entail relatively low costs to the host grass. Some E+ Arizona fescue plants in this population have no detectable peramine or other alkaloids (Faeth and Fagan, 2002). However, the low peramine levels also suggest few alkaloid-related benefits for the host. Deterrence of herbivory via alkaloids is considered the primary avenue for endophyte-related benefits to the host grass, which has been frequently demonstrated for E+ domesticated (e.g., Clay, 1988, 1990; Cheplick and Clay, 1988; Breen, 1994) and a few native grasses (e.g., Jones et al., 2000; Brem and Leuchtmann, 2001). Siegel and Bush (1996) and Rowan and Latch (1994) indicated that peramine alkaloid is generally deterrent and toxic to invertebrate herbivores, but only at levels greater than 3.0 μg/g.

Therefore, benefits of infection to the host related to herbivore deterrence via endophyte-related peramine seem improbable in E+ Arizona fescue. These low levels of peramine, and sometimes the complete absence of alkaloids, are not uncommon in many E+ native grasses (Leuchtmann et al., 2000). Lack of herbivore deterrence in E+ Arizona fescue has been demonstrated for vertebrate (Schulthess and Faeth, 1998; Saikkonen et al., 1999) and a suite of invertebrate (Lopez et al., 1995; Tibbets and Faeth, 1999; Saikkonen et al., 1999; Faeth, 2002) herbivores. Other possible benefits of peramine to the host include allelopathy and inhibition of pathogens or seed predators (e.g., Faeth and Bultman, 2002; Faeth, 2002), although preliminary tests (Faeth, et al., 2001; Faeth, 2002) and the low levels of peramine again suggest that these benefits to the host are unlikely.

E+ Arizona fescue, like many other *Neotyphodium*-infected native grasses (e.g., Leuchtmann et al., 2000), displays wide variation in alkaloid levels within and between populations (Faeth and Fagan, 2002). The observed variability of peramine within populations appears related to reshuffling of plant genotypes at each transmission event of a limited number of relatively static endophyte haplotypes.

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SEMIOCHEMICAL-MEDIATED FLIGHT RESPONSES OF SAP BEETLE VECTORS OF OAK WILT, *Ceratocystis fagacearum*

JOHN F. KYHL,[1] ROBERT J. BARTELT,[2] ALLARD COSSÉ,[2] JENNIFER JUZWIK,[3] and STEVEN J. SEYBOLD[1,*]

[1]*Departments of Entomology and Forest Resources*
University of Minnesota
1980 Folwell Avenue
St. Paul, Minnesota 55108, USA

[2]*USDA Agricultural Research Service*
National Center for Agricultural Utilization Research
Bioactive Reagents Research Unit
1815 N. University Street
Peoria, Illinois 61604, USA

[3]*USDA Forest Service*
North Central Research Station
1561 Lindig Avenue
St. Paul, Minnesota 55108, USA

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Abstract—The sap beetle, *Colopterus truncatus* (Coleoptera: Nitidulidae), is one of the primary vectors of the oak wilt pathogen, *Ceratocystis fagacearum*, in the north-central United States. Field behavioral assays utilizing various release rates and blends of three methyl-branched hydrocarbon aggregation pheromone components showed that flight responses of this beetle were similar in Illinois and Minnesota populations. In both locations, both sexes of the beetle responded synergistically to a combination of the three-component pheromone and fermenting whole-wheat bread dough. Further, *Colopterus truncatus* preferred a high release rate over a low release rate of the three-component blend. In both locations, the response of *C. truncatus* to a simplified version of the pheromone consisting of (2*E*,4*E*,6*E*)-3,5-dimethyl-2,4,6-octatriene (**1**) and (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene (**3**) was not significantly different from the response to the three-component blend. An experiment in Illinois with all possible combinations of the components demonstrated that the decatetraene (**3**) was the crucial component in the blend; of all treatments, the maximal response was elicited by **3** + dough. Chipped bark, phloem, and xylem from northern pin oak, *Quercus ellipsoidalis*, was not attractive to *C. truncatus* in Minnesota. During a weekly survey over two seasons in Minnesota, *C. truncatus* flew in response

* To whom correspondence should be addressed. E-mail: sseybold@tc.umn.edu

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to the three-component pheromone between early April and early July, with the maximum responses coming on May 4, 2000 and April 20, 2001. During both years, more than 98% of the beetles were trapped between April 14 and June 1. During the same survey, *Glischrochilus* spp. (Nitidulidae) flew during longer periods of the summer, particularly in 2001. The sex ratio of *C. truncatus* responding during all experiments was female-biased (1.8:1, female–male), which is characteristic of other male-produced coleopteran aggregation pheromones. Other sap beetles that play a minor role in the pathobiology of *C. fagacearum* also responded in experiments conducted in Minnesota. *Carpophilus brachypterus* Say was cross-attracted to the two- and three-component blends of the *C. truncatus* pheromone and dough, whereas two *Glischrochilus* spp. were attracted to all treatments that contained dough.

Key Words—*Colopterus truncatus*, oak wilt, *Ceratocystis fagacearum*, aggregation pheromone, Coleoptera, Nitidulidae, sap beetle, *Glischrochilus* spp., *Carpophilus brachypterus*, methy-branched hydrocarbons, monitoring, phenology.

INTRODUCTION

Oak wilt, *Ceratocystis fagacearum*, Bretz (Hunt), is a fungal disease that annually kills many oak trees (Fagaceae: *Quercus* spp.) across a large area in the eastern and north-central United States (Tainter and Baker, 1996). For example, by the mid-1990s, aerial photographs recorded 3000 oak wilt infection centers (areas) in Minnesota that contained 100,000 recently killed trees (French, 1995). This disease has had a significant effect on the vegetative composition of natural, periurban, and urban forests and is the disease of most concern to forest health specialists in 5 of the 22 states in which it occurs (Billings, 2000).

One way in which new oak wilt infection centers are established in the landscape is through the dispersal of oak wilt spores during the feeding and reproductive activities of sap beetles (Coleoptera: Nitidulidae). These insects are attracted to and aggregate on oak wilt fungal fruiting tissues (mats) that occur on oaks recently killed by the pathogen (Lin and Phelan, 1992; Cease and Juzwik, 2001). The beetles feed, mate, and oviposit on the mats from which oak wilt fungal propagules are ingested and accumulate on the cuticular surfaces of the adults and larvae (Curl, 1955; Jewell, 1956; Juzwik, 1983). Spore-laden sap beetles also aggregate, feed, mate, and oviposit in sap fluxes associated with fresh wounds on uninfected oak trees, thereby propagating the disease (Jewell, 1956). The response of sap beetles to wounded oak tissue can be remarkably rapid; some species may be attracted to experimentally created wounds within 15 min (J.J., personal observation). In one study, up to 78% of sap beetles that responded to wounds carried viable oak wilt spores in or on their bodies (Juzwik et al., 1999). Behaviors associated with exposure to inoculum and the visitation of fresh wounds have led to the identification of two sap beetles, *Colopterus truncatus* (Randall) and *Carpophilus sayi* (Parsons),

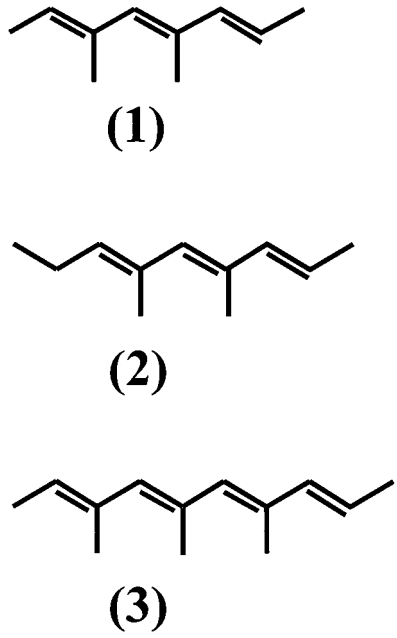


FIG. 1. Hydrocarbon components of the male-produced *Colopterus truncatus* aggregation pheromone: (**1**) (2*E*,4*E*,6*E*)-3,5-dimethyl-2,4,6-octatriene, (**2**) (2*E*,4*E*,6*E*)-4,6-dimethyl-2,4,6-nonatriene, and (**3**) (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene.

as the primary vectors of oak wilt in the north-central United States (Juzwik et al., 1999; Cease and Juzwik, 2001).

In order to improve our understanding of the life history of *C. truncatus*, we investigated the response of this insect and associated sap beetles to semiochemicals in oak forests of Illinois and Minnesota. Little is known about the chemical ecology of the response of *C. truncatus* to oak wilt mats or to wounded oaks in the field. However, a three-component, male-produced aggregation pheromone was recently isolated and identified from *C. truncatus* (Cossé and Bartelt, 2000). Three methyl-branched hydrocarbons, (2*E*,4*E*,6*E*)-3,5-dimethyl-2,4,6-octatriene (**1**), (2*E*,4*E*,6*E*)-4,6-dimethyl-2,4,6-nonatriene (**2**), and (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene (**3**) (Figure 1), elicited electroantennographic activity from male and female antennae, and when combined with fermenting whole-wheat bread dough as a trap bait, synthetic hydrocarbon **2**, the combination of **1** and **3**, and the combination of **1**, **2**, and **3**, all elicited flight responses from both sexes that significantly exceeded the response to dough alone (Cossé and Bartelt, 2000). The nonatriene (**2**) was the most abundant of the three components; volatiles collected from males indicated emission rates of 1.8:100:3.3 for **1**, **2**, and

3, respectively. However, Cossé and Bartelt (2000) did not unequivocally establish the relative significance of the components for the flight response of *C. truncatus*, particularly in light of the differences in volatility of components **2** and **3** after they had been loaded on rubber septa for slow release in the field.

In this study, we report new information on the chemical ecology of *C. truncatus* and other sap beetles associated with oak wilt. The specific goals of this study were to: (1) determine and compare the responses of *C. truncatus* to its pheromone at two different locations in the north-central United States; (2) optimize the blend and release rate of the *C. truncatus* synthetic aggregation pheromone components and explore the potential of specific synergy of the pheromone with oak tissue; and (3) determine the flight period of *C. truncatus* in oak forests in Minnesota to more clearly define the high risk seasonal period for overland transmission of oak wilt.

METHODS AND MATERIALS

Semiochemicals and Baits. As attractants, we used the synthetic aggregation pheromone components for *C. truncatus* [**1**, **2**, and **3** (Cossé and Bartelt, 2000) (Figure 1)] combined with fermenting whole-wheat bread dough or oak chips. Our standard attractant for *C. truncatus* consisted of dough in a plastic cup (see below) and 3.5, 500, and 280 μg each of the all *E* isomers of **1**, **2**, and **3**, respectively, applied in hexane to a rubber septum. Solution concentrations were quantified by capillary GC using an internal standard, and all pheromone components were formulated at the USDA-ARS laboratory in Peoria (Cossé and Bartelt, 2000). With these initial amounts of **1**, **2**, and **3** loaded on a septum, laboratory analyses have indicated that the proportions released during the first day from a trap are very close to those emitted by male beetles (Cossé and Bartelt, 2000). In two experiments, the treated septa were placed within 1.5 ml polyethylene Eppendorf microcentrifuge tubes each with a single pinhole in the lid (low release rate). Otherwise, the treated septa were pinned directly to the trap (high release rate).

The synthetic compounds had ca. 80% chemical purity and after passage through silica gel with hexane to remove polar contaminants, the remaining impurities were small amounts of *Z* isomers. The *Z* isomers result both as artifacts of synthesis and from slow isomerization of the *E* isomers, and there is presently no simple, large-scale method available for their removal. However, with other nitidulids, small amounts of *Z* isomers have not significantly reduced trap catch (Bartelt, 1999).

When compound **3** is synthesized (Bartelt et al., 1990), about 1–2% of the product consistently is compound **1** (which arises because a Wittig-Horner condensation does not quite go to completion). When a 100:1 mixture of **3** and **1** is applied to a rubber septum, **3** and **1** are released with an initial ratio of about 5:1 instead of 100:1 because of the impurity and because of their different boiling

points (Cossé and Bartelt, 2000). Thus, synthetic **3** on a rubber septum will deliver both **3** and **1** into the air at approximately the same ratio as from the male beetles (3.3:1.8). No complication of this sort arises with **2** because neither **3** nor **1** are produced in its synthesis, nor is **2** produced in the synthesis of **1** or **3**.

For one experiment (IV), samples of **1**, **2**, and **3** were purified further so that they could be tested individually and in all possible combinations. Synthetic **3** was purified by HPLC on a $AgNO_3$-coated silica column (Heath and Sonnet, 1980), using 0.5% 1-hexene in hexane as the solvent. The procedure removed all traces of **1** and a substantial fraction of the *Z* isomers [final chemical purity 90%, by capillary GC, instrumentation as in Cossé and Bartelt (2000)]. The major triene **2** was similarly purified to >90% by HPLC; the purity of minor triene **1** was 81%. After HPLC purification, this sample was free of compounds **2** and **3**.

Fermenting whole-wheat bread dough (dough) was used in these experiments because it is an attractive food bait to a variety of nitidulid species and because it has also been shown to act as a synergist for all nitidulid pheromones tested (Bartelt, 1997). Dough was prepared in the laboratory between 2 and 6 hr before use and consisted of whole-wheat flour, table sugar, water, and baker's yeast (3:1:3–4:0.1). Approximately 10–15 ml of dough was placed in a 35-ml plastic cup, and the cup was placed in the trap.

To test for the possibility of attraction of *C. truncatus* to volatiles produced by oaks, tissue was collected from a healthy northern pin oak, *Quercus ellipsoidalis* (E. J. Hill), in mid-April 2001. Live branches (<6 cm diam.) were cut and chipped using a chipper/shredder. All branch chips were mixed together and stored in heavy plastic bags in a −55°C freezer until they were placed in the field.

Traps and Field Sites. All studies used wind-oriented funnel traps (Dowd et al., 1992), which were hung from tree branches or posts ca. 1–1.5 m above the ground. Traps were placed at least 10 m apart. In one experiment, the traps were slightly modified to hold approximately 300 ml of oak chips. In Illinois, all studies were conducted in a single mixed hardwood forest near East Peoria (40.7°N latitude, 89.8°W longitude), while in Minnesota, four separate northern red/white oak (*Quercus rubra* L./*Quercus alba* L.) forests near Minneapolis-St. Paul were used as field sites. All four Minnesota field sites were separated by at least 5 km: Carlos Avery Wildlife Management Area (45.4°N latitude, 93.0°W longitude), a private residence in Columbus township (45.2°N latitude, 93.1°W longitude), Katherine Abbott Park in Mahtomedi (45.1°N latitude, 93.0°W longitude), and a private residence in Lake Elmo (45.0°N latitude, 92.9°W longitude). If more than one block was located at a site, blocks were separated by at least 100 m.

Field Bioassays. Six experiments were conducted in this study. They took place in Illinois in 2000 and in Minnesota in 2000 and 2001.

Experiment I. A study was conducted from late March to mid-April, 2000 in Illinois to establish the importance of the dough coattractant and to determine which of two pheromone release rates was most attractive to *C. truncatus*. The

experiment consisted of: (1) a high release rate of **1** + **2** + **3**, (2) a low release rate of **1** + **2** + **3**, (3) dough, (4) a high release rate of **1** + **2** + **3** + dough, (5) a low release rate of **1** + **2** + **3** + dough, and (6) an unbaited control. The experiment was initiated on March 24 with two completely randomized blocks (six traps per block), and the traps were emptied semiweekly until April 18. Treatment locations were rerandomized within blocks and dough baits were replaced after each trap was emptied. Pheromone baits were replaced weekly, but the replacement was staggered so that it was done in just one of the blocks after each trap check. Thus, after March 28 pheromone baits of two ages were always present, and in principle, changes in bait attractiveness over time would not be completely confounded with day effects.

Experiment II. Experiment I (Illinois) was duplicated in 2000 in Minnesota with the additional goal of monitoring the seasonal flight periodicity of *C. truncatus*. The same six treatments and the same trap type from experiment I were used. Experiment II was initiated on April 14 in a completely randomized block design in each of four field sites. Traps were emptied, rerandomized, and rebaited weekly until November 3. Traps were not baited in a staggered fashion as described in experiment I.

Experiment III. In 2001, the response to different treatments and the seasonal flight periodicity of *C. truncatus* were again monitored in Minnesota. This experiment consisted of: (1) a high release rate of **1** + **2** + **3** + dough, (2) dough, and (3) an unbaited control. The experiment was initiated on April 5 and continued until November 9. Sites, rebaiting, and rerandomization schedules were the same as in 2000.

Experiment IV. In 2000, in a second experiment in Illinois, the effects of **1**, **2**, and **3** on the response of *C. truncatus* were studied. In this case, the compounds were released individually and in all possible combinations. The amounts of each component loaded on the septa were 3.5, 500, and 280 μg, respectively, no matter what combination was tested. Based on the results of experiment I, high release rates of **1**, **2**, and **3** were always combined with dough. The experiment consisted of: (1) **1** + dough, (2) **2** + dough, (3) **3** + dough, (4) **1** + **2** + dough, (5) **1** + **3** + dough, (6) **2** + **3** + dough, (7) **1** + **2** + **3** + dough, and (8) dough alone control. The experiment was established as two completely randomized blocks, each with eight traps, in the same woodlot used for experiment I. Traps were set on posts at a height of 1 m and were baited fresh at ca. 07:00 AM on mornings with a favorable weather forecast (i.e., temperature $>15^{\circ}$C, with minimal wind and cloud cover). As peak flight activity of *C. truncatus* occurs in mid- to late afternoon (R.J.B., personal observation), traps were emptied either near dusk or early the following morning. Treatment locations were rerandomized within blocks each day. Seven replications of the experiment were run between April 21 and May 5, 2000, and each replication was of short duration to ensure that the component ratios and emission rates of the pheromone components would be essentially stable throughout the study.

Experiment V. Based on the results of experiment IV in Illinois, an experiment was conducted in 2001 in Minnesota (same four sites) to compare responses of *C. truncatus* to the high release rate of **1** + **2** + **3** and to the high release rate of **1** + **3**. As in experiment IV, the amounts of **1**, **2**, and **3** loaded on the septa were always 3.5, 500, and 280 μg, respectively. However, in this instance, component **3** also consisted of 1–2% of component **1**. The experiment consisted of: (1) **1** + **2** + **3** + dough, (2) **1** + **3** + dough, (3) dough, and (4) an unbaited control. Traps were hung from trees at a height of 1.5 m, baited fresh between 07:00 and 10:00 AM on days with a favorable weather forecast (as above), and emptied 24 hr later. This experiment was conducted for eight days between April 28 and May 31.

Experiment VI. An experiment was conducted in 2001 in Minnesota to compare responses of *C. truncatus* to oak chips (i.e., their volatiles) with responses to oak chips plus the high release rate of **1** + **3**. The experiment consisted of: (1) **1** + **3** + 300 ml oak chips, (2) 300 ml oak chips, and (3) an unbaited control. The experiment was set up as completely randomized blocks in each of the four previously described sites. Traps were placed, emptied, rebaited, and rerandomized on the same schedule and dates as in experiment V. The oak chips were replaced daily, and the previous day's chips were always moist when they were removed from the traps, increasing the likelihood that volatiles were still being released.

Statistical Analyses. Data were tabulated by species and sex, with the exception of experiment II, in which the sexes of responding *C. truncatus* were not determined. Nitidulid beetles other than *C. truncatus* were also tallied in the Minnesota experiments. Data from all experiments were analyzed by Friedman's nonparametric two-way ANOVA. Data from experiment IV also were analyzed by standard two-way ANOVA, after transformation of the trap-catch counts by log $(X + 1)$, because several linear contrasts were of particular interest. When F statistics were significant, means were compared using LSD (Bonferroni's method) with an experiment-wise $\alpha = 0.05$. Since trap catches of *C. truncatus* dropped off dramatically after mid-June in Minnesota, statistical analyses of responses of this species in 2000 and 2001 were only carried out on data collected during the mid-April to the mid-June periods. In some cases, analyses of trap catches of other nitidulid species spanned the entire trapping season. Voucher specimens from Minnesota trap catches were deposited in the University of Minnesota Insect Collection.

RESULTS

Experiment I. The high release rate of **1** + **2** + **3** + dough was the most attractive treatment to *C. truncatus* (Friedman's $\chi^2 = 367.1$, $df = 5$, $P < 0.001$; Figure 2A). The high release rate of **1** + **2** + **3** and dough were clearly synergistic; without the dough, *C. truncatus* did not respond to the high release rate of **1** + **2** + **3**, and dough alone attracted about one sixth the mean number of *C. truncatus* that responded to the combined treatments. There was no difference

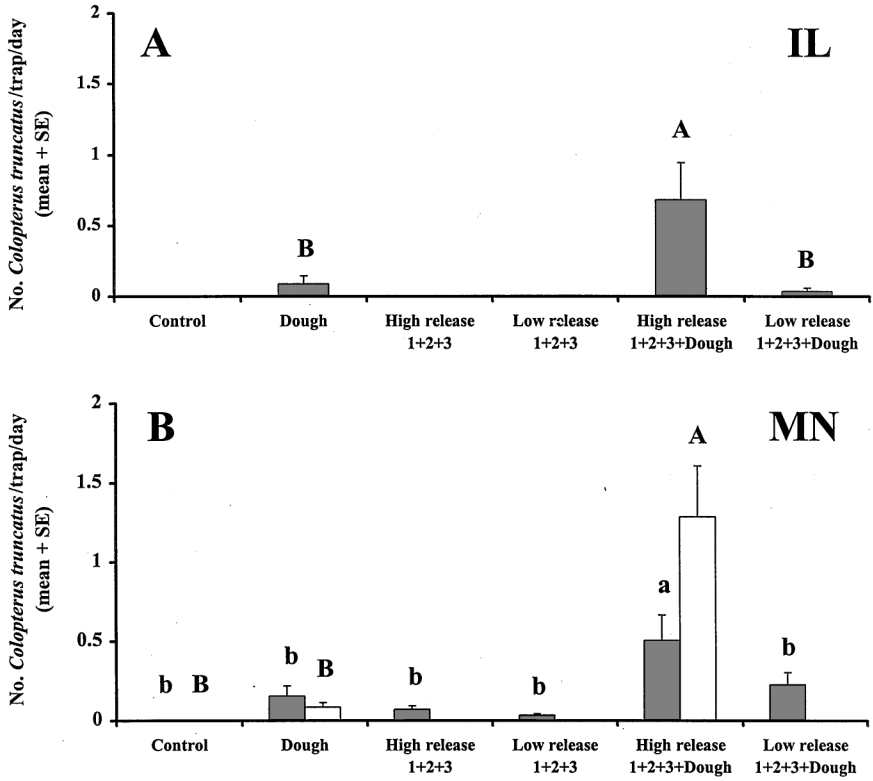


FIG. 2. Flight response of *Colopterus truncatus* to high and low release rates of synthetic aggregation pheromone components **1** + **2** + **3**, with and without dough, from (A) March 24 to April 18, 2000 in Illinois (experiment I) and from (B) April 4 to June 15, 2000, (dark bars) and April 5 to June 15, 2001 (open bars) in Minnesota (experiments II and III). Responses to treatments are significantly different if histogram bars within a year are marked with different letters.

between the responses of *C. truncatus* to dough alone and to dough combined with **1** + **2** + **3** released at the low rate. Overall, trap catches were too low to take advantage of the information from staggered pheromone replacement. Both sexes of *C. truncatus* (31 females and 21 males) responded in this experiment, and the sex ratio was not significantly different among treatments ($\chi^2 = 0.07$, $df = 5$, $P = 0.97$). No *C. truncatus* were collected in control traps.

Experiments II and III. *Colopterus truncatus* responded to the two release rates of its aggregation pheromone in Minnesota as it did in Illinois (Figure 2B). In 2000, the high release rate of **1** + **2** + **3** combined with dough was clearly the most attractive treatment to *C. truncatus* (Friedman's $\chi^2 = 3{,}414.9$, $df = 5$, $P < 0.001$;

Figure 2B). In experiments II, III, V, and VI, there were no significant effects for site or the site × treatment interaction for any of the taxa that responded. In experiments I and IV, only one site was used. As in Illinois, the responses of *C. truncatus* to dough alone and to the high release rate of **1** + **2** + **3** alone were low and did not differ significantly from one another or from the response to the unbaited trap. However, when the dough and high release rate of aggregation pheromone were combined, the response increased more than threefold above the response to the high release rate of **1** + **2** + **3** alone, indicating that the treatments were synergistic. Both treatments involving the low release rate of **1** + **2** + **3** elicited responses that were not significantly different from the response to the unbaited trap. However, when the low release rate of **1** + **2** + **3** was combined with the dough, the response was only exceeded by the comparable high release rate treatment. In 2001, the high release rate of **1** + **2** + **3** + dough was the most attractive treatment to *C. truncatus* (Friedman's $\chi^2 = 363.7$, $df = 2$, $P < 0.001$; Figure 2B). Although the numbers of trapping days were nearly identical, overall *C. truncatus* trap catch was nearly twice as great in 2001 (385) when compared to 2000 (206). Both sexes of *C. truncatus* responded in 2001 (the only year sex ratio data were collected); 247 females and 138 males were captured, and the sex ratio did not vary across treatments ($\chi^2 = 0.28$, $df = 2$, $P = 0.59$). No *C. truncatus* were collected in control traps during either year.

Two other nitidulid beetles, *Glischrochilus quadrisignatus* (Say) and *G. fasciatus* (Olivier), responded to the treatments in experiments II and III in a different way than did *C. truncatus* (Figure 3). In both years, these species responded to all traps containing dough at a higher level than those without dough (2000: Friedman's $\chi^2 = 3{,}722$, $df = 5$, $P < 0.001$; 2001: Friedman's $\chi^2 = 588.1$, $df = 2$, $P < 0.001$). No synergism was found, and there was no discernible response to either release rate of **1** + **2** + **3** alone. Hence, there was no evidence for cross-attraction of *G. quadrisignatus* and *G. fasciatus* to the pheromone of *C. truncatus*. The total pooled trap catch for *G. quadrisignatus* and *G. fasciatus* was nearly twice as great in 2000 (542) when compared to 2001 (337). Two other sap beetles, *Carpophilus sayi*, and *Epuraea corticina* Erichson were trapped in experiments II and III, but not in sufficiently high enough numbers to analyze.

Seasonality in Experiments II and III. There was a definite seasonality to the flight activity of *Colopterus truncatus* in Minnesota. In both years, adult activity was initiated in mid- to late April, peaked between late April and early May, and largely disappeared by early and mid-June (Figure 4A). Continued monitoring until November caught only three additional *C. truncatus*, one on September 15, 2000, and one each on August 24 and 31, 2001. The maximum flight responses came on May 4 (2000) and April 20 (2001), and during both years, more than 98% of the beetles were trapped between April 14 and June 1.

The seasonal pattern of flight activity of *G. quadrisignatus* and *G. fasciatus* (both species pooled) was different than *C. truncatus* (Figure 4B). In 2000, these

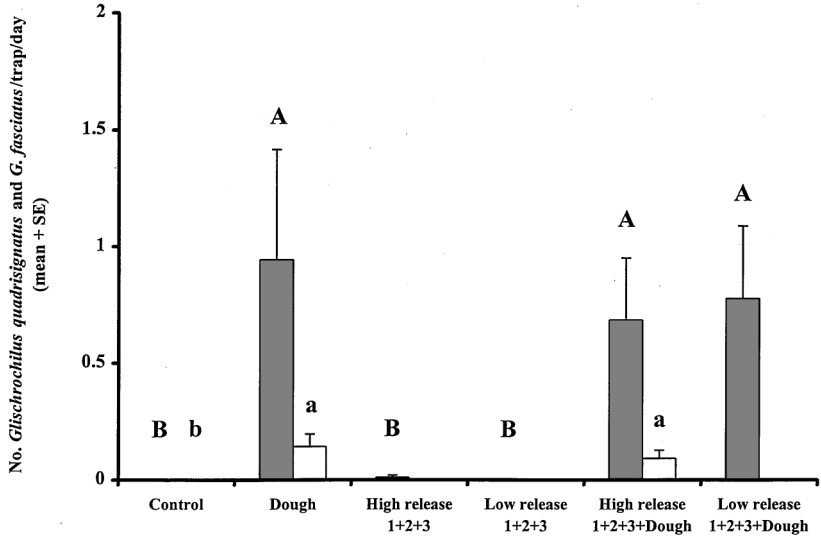


FIG. 3. Pooled flight responses of *Glischrochilus quadrisignatus* and *G. fasciatus* to high and low release rates of synthetic *Colopterus truncatus* aggregation pheromone components **1** + **2** + **3**, with and without dough, from April 4 to June 15, 2000 (dark bars) and April 5 to June 15, 2001 (open bars) in Minnesota (experiments II and III). Responses to treatments are significantly different if histogram bars within a year are marked with different letters.

species were abundant in the spring and their presence continued at a low, but discernible level throughout the rest of the year, showing a pattern somewhat similar to *C. truncatus*. In 2001, there was no large spring peak, and the abundance varied throughout the remainder of the year, peaking in mid- to late July. While there was a large amount of variation in trap catches during both years of the study, *G. quadrisignatus* and *G. fasciatus* were generally more abundant than *C. truncatus*, and they did not show the same distinct limited seasonality in Minnesota.

Experiment IV. The effect of all possible combinations of **1** + **2** + **3** on *C. truncatus* in Illinois, showed a strong treatment effect, using either the Friedman's nonparametric two-way ANOVA (Friedman's $\chi^2 = 2{,}104.2$, $df = 7$, $P < 0.001$) or the standard two-way ANOVA with data transformed using $\log(X + 1)$ ($F = 21.7$, $df = 7{,}49$, $P < 0.001$) (Figure 5). The day × treatment interaction was not significant ($F = 1.3$, $df = 42{,}49$, $P < 0.185$), indicating that relationships among treatments were fairly consistent from day to day. Individual block effects were allowed to vary from day to day in the analysis (i.e., the day × block interaction was fitted); this precaution allowed for the possibility that the *C. truncatus* population density changed in different ways over

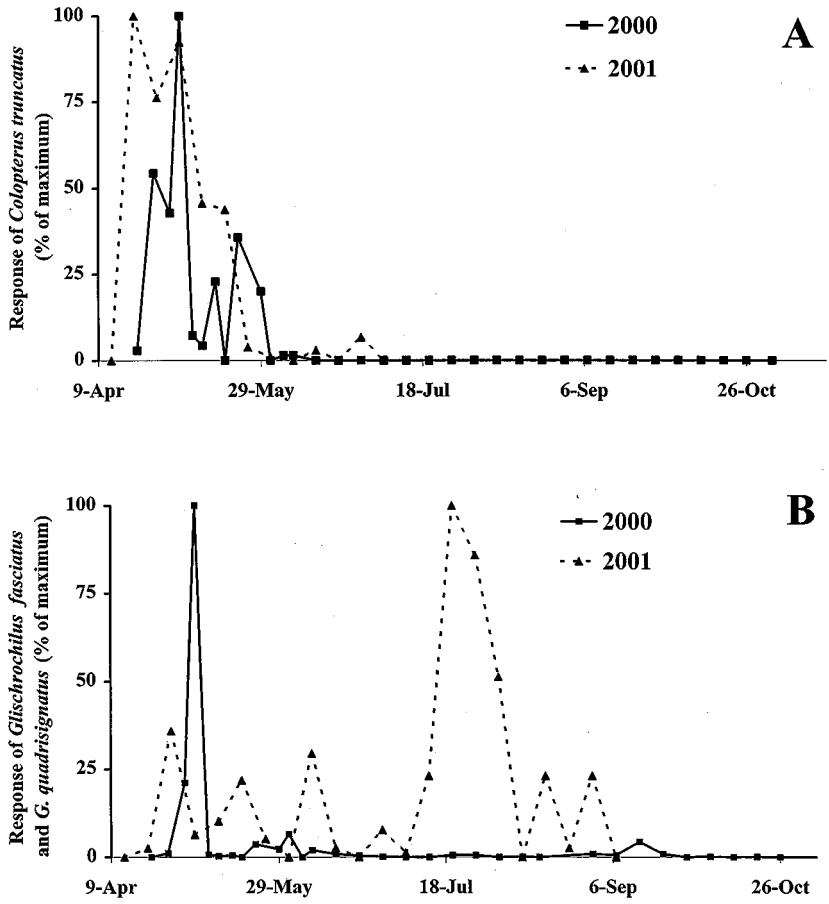


FIG. 4. Relative seasonal flight responses of (A) *Colopterus truncatus* to all treatments in experiments II and III from April 4 to November 3, 2000, and April 5 to November 9, 2001, in Minnesota and (B) *Glischrochilus quadrisignatus* and *G. fasciatus* to all treatments in experiments II and III from April 4 to November 3, 2000, and April 5 to November 9, 2001, in Minnesota. Of the 591 *C. truncatus* trapped in 2000 and 2001, only three beetles were trapped after early July (September 15, 2000; and August 24 and 31, 2001—both females). Maximum trap catch per week in 2000 was 40 beetles (May 4), whereas maximum trap catch per week in 2001 was 97 beetles (April 20). For *Glischrochilus* spp., maximum trap catch per week in 2000 was 136 beetles (May 11), whereas the maximum trap catch per week in 2001 was 76 beetles (July 18).

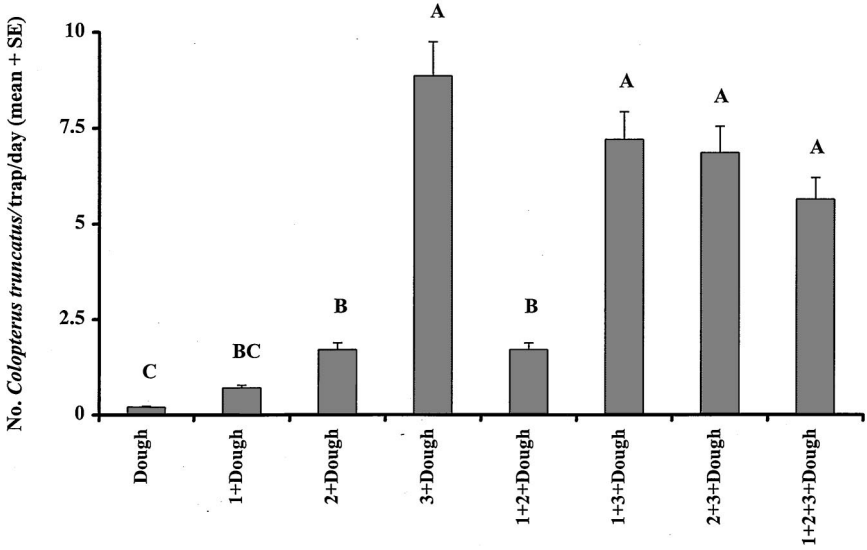


FIG. 5. Flight response of *Colopterus truncatus* to high release rate of all possible combinations of synthetic aggregation pheromone components **1**, **2**, and **3** with dough (experiment IV) in Illinois. Responses to treatments are significantly different if histogram bars are marked with different letters.

time in different areas of the site. The dominant effect in the experiment was that trap catches were dramatically higher whenever compound **3** was present in the bait (the linear contrast for the presence versus absence of **3** was significant, $F = 135.7$, $df = 1,49$, $P < 0.001$). On the other hand, the corresponding contrasts for **1** and **2** were not significant ($F = 0.0009$, $df = 1,49$, $P = 0.973$; $F = 1.932$, $df = 1,49$, $P = 0.170$, respectively), although the response to **2** plus dough was nominally more attractive than and significantly different from the response to the dough control (Figure 5). The higher-order effects for interactions among treatments were significant ($F = 3.595$, $df = 4,49$, $P = 0.012$), but these were minor when compared with the contrast for **3**. That the interaction was significant at all was due to catches for **3** being somewhat lower when **2** was also present versus when **2** was absent, for unknown reasons. This trend was not significant when the means were considered just two at a time with LSD tests. Overall, 291 female and 171 male *C. truncatus* were captured in experiment IV. The sex ratio did not differ significantly among treatments ($\chi^2 = 6.2$, $df = 7$, $P = 0.52$).

Experiment V. In Minnesota, the responses of *C. truncatus* to the high release rate of $\mathbf{1} + \mathbf{2} + \mathbf{3} +$ dough and to the high release rate of $\mathbf{1} + \mathbf{3} +$ dough were not significantly different (Friedman's $\chi^2 = 1{,}453.2$, $df = 3$, $P < 0.001$; Figure 6A). Both pheromone-containing treatments were more attractive than the

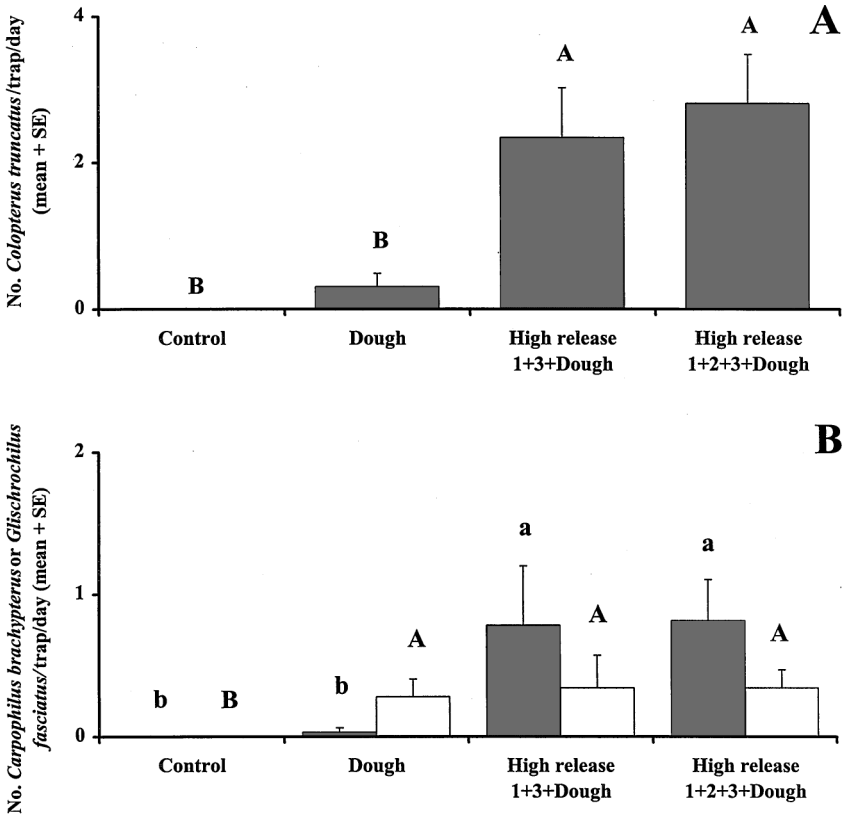


FIG. 6. Flight responses of (A) *Colopterus truncatus* and (B) *Carpophilus brachypterus* (shaded bars) and *Glischrochilus fasciatus* (open bars) to high release rate of synthetic *Colopterus truncatus* aggregation pheromone components **1** + **2** + **3** + dough, high release rate of **1** + **3** + dough, dough alone, and unbaited control in Minnesota (experiment V). Responses to treatments are significantly different if histogram bars within a species are marked with different letters.

dough alone or unbaited control treatments. Of the 182 beetles in the traps, there were 117 females and 65 males, and the sex ratio was not significantly different among treatments ($\chi^2 = 0.48$, $df = 3$, $P = 0.79$).

Two other nitidulids, *Carpophilus brachypterus* (Say) (52 beetles caught) and *Glischrochilus fasciatus* (31 beetles caught), also responded to treatments in experiment V (Figure 6B). *Carpophilus brachypterus* responded to the treatments similarly to *Colopterus truncatus* (albeit at lower levels), with a significant and similar response to both pheromone–dough combinations (Friedman's $\chi^2 = 813.8$, $df = 3$, $P < 0.001$; Figure 6B). *Glischrochilus fasciatus* responded

to the treatments differently than *C. truncatus* and *C. brachypterus*. With *G. fasciatus*, there were no significant differences among the responses to traps containing dough (Friedman's $\chi^2 = 269.1$, $df = 3$, $P < 0.001$; Figure 6B). Although some *G. quadrisignatus* were collected in this study, the catches (18 beetles) were too low to discern any pattern in the response.

Experiment VI. The high release rate of **1** + **3** + oak chips was the most attractive treatment to *C. truncatus* (Friedman's $\chi^2 = 147.0$, $df = 2$, $P < 0.001$). Overall trap catches in this experiment were quite low: no beetles were caught in either the control or oak chips-alone treatments, and more than 75% of the traps baited with **1** + **3** + oak chips were empty. Of the 16 *C. truncatus* caught in the traps baited with **1** + **3** + oak chips, there were 12 females and 4 males. No other sap beetles were caught in this experiment.

DISCUSSION

Both Illinois and Minnesota populations of *C. truncatus* responded similarly to the low- and high-release rate formulations of **1** + **2** + **3** with dough and to dough alone. Both populations preferred the high release rate formulation. Since the low release rate pheromone formulation keeps the ratios of emitted volatiles constant for a longer period of time than the high release rate (i.e., the unenclosed septum) formulation (R.J.B., unpublished data), our trapping results suggest that the sustained emission of the constant ratio of pheromone components is not the most important factor in the attraction of *C. truncatus*. Rather, they suggest that the total amount of components emitted was the most important factor in trap catch.

In both Illinois and Minnesota populations, there was no difference in the response to the combination of **1** + **2** + **3** with dough or the combination of **1** + **3** with dough. In contrast to earlier work in Illinois on *C. truncatus* (Cossé and Bartelt, 2000), this study showed directly in Illinois, and by inference in Minnesota, that component **2** was not necessary for, and in fact perhaps reduced, the attraction of *C. truncatus*. Even though component **2** was the most abundantly produced of the three male-specific hydrocarbons investigated in this study, it was component **3** that dominated the response. Component **3** had not been tested alone in previous behavioral studies, but it did show considerable electrophysiological activity when presented alone to male and female antennal preparations (Cossé and Bartelt, 2000). Although there were no apparent differences in the responses of Illinois and Minnesota populations of *C. truncatus* to various release rates and blends of the aggregation pheromone, there may be differences in the seasonal pattern of response to aggregation pheromone (see below).

Since *C. truncatus* and other nitidulids have a relatively rapid and strong response to fresh wounds in oak trees (Juzwik et al., 1999; J.J., personal observation), we hypothesized that the volatiles produced by oak tissue would synergize the *C. truncatus* aggregation pheromone. Based on the evidence shown here

(experiment VI), volatiles produced from chipped oak tissue are not attractive, and these volatiles likely do not synergize the pheromone. Trap catches in experiment VI were quite low, and are probably explained by a low-level response to the pheromone without the dough synergist as observed in experiments I and II. The oak tissue in our experiments might not have been attractive to *C. truncatus* because the process of chipping might produce different volatiles (or different amounts of volatiles) than those normally encountered by nitidulids when searching for fresh sap-laden wounds on living trees. Further work is needed to identify and characterize the volatiles of wounded oaks, how they change over time, and when they are most attractive to nitidulids searching for feeding, mating, and ovipositional sites. Preliminary chemical analyses, however, show that volatiles from individuals within an oak species are quite variable and suggest that this work will be difficult (R.J.B., unpublished data).

Since sap beetles aggregate on oak wilt mats (Cease and Juzwik, 2001), it is possible that oak wilt mat volatiles synergize the aggregation pheromone emitted by *C. truncatus* and other species while on mat tissue. Volatiles from laboratory cultures of oak wilt have been described and are attractive to sap beetles (*C. truncatus* was not tested) in wind-tunnel bioassays (Lin and Phelan, 1992). Further study on the relationship between oak wilt mat volatiles and nitidulid pheromones will be conducted when a stable *Ceratocystis fagacearum* volatile formulation is available for field tests.

The distinct seasonality shown by *C. truncatus* in our study is very important in understanding and managing the overland spread of oak wilt. Peak production of oak wilt mats (Campbell and French, 1955) and the window of peak susceptibility to infection (Prey and Kuntz, 1995) both occur during the spring in Minnesota (Juzwik et al., 1985). Our results show that in Minnesota, *C. truncatus* is attracted to pheromone + dough-baited traps in April, May, and June, but very few *C. truncatus* are caught in the same traps with the same baits later in the summer and in the fall. Skalbeck (1976), when trapping with fermenting dough and *Ceratocystis fimbriata* baits over two years in three Minnesota forest types, also found *C. truncatus* only in the spring and early summer, with the peak abundance in April and May. In another instance in Minnesota, of 40 *C. truncatus* trapped in response to combinations of dough and (2*E*,4*E*,6*E*,8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-decatetraene, 95% of the trap catch flew before July 6 (one male and one female were trapped in September) (R.J.B., unpublished data). However, McMullen and Shenefelt (1961), Levesque and Levesque (1992), Dowd and Nelsen (1994), and Cossé and Bartelt (2000) have all reported trapping *C. truncatus* in flight during the fall to various stimuli in Wisconsin, Quebec (Canada), Illinois, and Illinois, respectively. Climatic differences between Minnesota and these other north-central areas may influence the life history, and, specifically, the flight behavior of *C. truncatus*.

Sampling *C. truncatus* from oak wilt mats in Minnesota has revealed that it is univoltine. Adults disperse in flight for mating in the spring and early summer,

larvae only occur in the mats in spring and early summer, and adult densities are approximately eight times higher in the mats in the fall than in the spring (J.F.K., unpublished data). As the fall cohort of oak wilt mats is newly formed each fall, it is likely that *C. truncatus* has flown to the mats at this time. These adults are also likely to feed on these mats and are less likely to disperse from them again through flight. Rather, they are more likely to drop to the litter layer on the forest floor to overwinter. Further work in Minnesota needs to be done to determine whether *C. truncatus* flies selectively in the spring and early summer in response to synergized synthetic pheromone (as a mating and feeding response) and selectively in the early fall to mat odors (as a feeding and preoverwintering response). Alternatively, *C. truncatus* may fly in response to pheromone in the fall, but at much lower densities or it may reach the mats in the fall by crawling to them. It should be noted that the seasonally restricted flight behavior of *C. truncatus* in Minnesota is not unique among sap beetles. Some species in the genus *Carpophilus* also show strong seasonal flight activity periods in California (Bartelt et al., 1994), South Carolina (Bartelt et al., 1995), and Australia (James et al., 1994), while species of *Colopterus* and *Cryptarcha* show a similar seasonal pattern in Texas (Appel et al., 1986).

As with other nitidulid pheromones (Bartelt, 1997; Zilkowski and Bartelt, 1999), the *C. truncatus* aggregation pheromone is cross-attractive to other species. In our experiments in Minnesota, as was the case in previous work in Illinois (Cossé and Bartelt, 2000), *Carpophilus brachypterus* responded to the synergized *Colopterus truncatus* pheromone. Since the decatetraene (**3**) is the main component of the aggregation pheromone of *Carpophilus brachypterus* (Williams et al., 1995), the response of *Carpophilus brachypterus* is most likely due to pheromone component similarity, reflecting the close phylogenetic relationship between the genera *Colopterus* and *Carpophilus* within the subfamily Carpophilinae (Downie and Arnett, 1996). The effect could also be interpreted as a kairomonal response. *Carpophilus brachypterus* may benefit by locating wounds in oaks or mats originally colonized by *Colopterus truncatus* and signaled by aggregation pheromone production by *C. truncatus*. Since *Carpophilus brachypterus* has been collected from oak wounds (Vogt, 1950; Norris, 1956), this chemically guided ecological relationship seems plausible. However, our review of the literature and a reexamination of summary data in Skalbeck (1976) suggests that *C. brachypterus* has not been collected to date from oak wilt mats (T.C. Skalbeck, personal communication). *Glischrochilus quadrisignatus* and *G. fasciatus* (subfamily Cryptarchinae) responded equally to all treatments containing dough. Thus, these species respond to food volatiles (Lin and Phelan, 1991), but do not show a kairomonal response to the *C. truncatus* aggregation pheromone.

One interesting outcome from these experiments is that female *C. truncatus* consistently responded to the *C. truncatus* aggregation pheromone in both Illinois and Minnesota at nearly twice the rate that males did. The sex ratio of *C.*

truncatus trapped in all experiments from both locations was approximately 1.8:1 (female–male) (Minnesota 1.8:1; Illinois 1.7:1). In the previous study with aggregation pheromone components of *C. truncatus* in Illinois, Cossé and Bartelt (2000) also reported female-biased sex ratios between 1.1 and 1.7:1. In other studies with sap beetles, Petroski et al. (1994) recorded a sex ratio of 1.2:1 in *Carpophilus obsoletus* Erichson responding to its aggregation pheromone in the field, whereas Bartelt et al. (1994) reported sex ratios "close to" 1:1 in five *Carpophilus* spp. and in *Haptoncus luteolus* (Erichson) responding to aggregation pheromones in the field. In the latter study, the sex ratios for four of the *Carpophilus* spp. and for *H. luteolus* were nearly 1:1 in date fruits, which are the hosts colonized by the beetles. The sex ratio of a sample of *C. truncatus* collected in oak wilt mats in Minnesota was also nearly 1:1 (1.1:1, $N = 128$; J.F.K., unpublished data).

In other coleopteran aggregation pheromone systems with a male-produced attractant, the sex ratio of beetles responding behaviorally to pheromone or the magnitude of the electrophysiological response to pheromones also tends to be female biased. Indeed, aggregation pheromones may, in part, serve a sex pheromone function (Borden, 1985). Female-biased responses have been reported in bark beetles (Scolytidae), e.g., the California five-spined ips, *Ips paraconfusus* Lanier (Wood et al., 1967, 1968; Byers, 1983), and the pine engraver, *Ips pini* (Say) (Seybold et al., 1992; Miller et al., 1990, 1995, 1996; Miller and Borden, 1990, 2000; Miller, 2000) and in grain beetles (Bostrichidae, Cucujidae, and Silvanidae), e.g., the larger grain borer, *Prostephanus truncatus* (Horn) (Birkenshaw and Smith, 2000), the rusty and flat grain beetles, *Cryptolestes ferrugineus* (Stephens) and *C. pusillus* (Schönherr) (Chambers et al., 1990), and the saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.) (White and Chambers, 1989). See Millar et al. (1985 a,b) and Pierce et al. (1988) for counter-examples with grain beetles. In the initial case of the isolation and identification of an aggregation pheromone from an insect (*I. paraconfusus*, Wood et al., 1967, 1968), the sex-specific production and the female-biased responses caused the investigators to view the signals as sex attractants. Since female *C. truncatus* are attracted at a greater rate than males in our study, this nitidulid example lends additional support to the hypothesis that male-produced aggregation pheromones evolved secondarily from their initial roles as sex pheromones to attract females (Raffa et al., 1993; Phillips, 1997; Plarre and Vanderwel, 1999).

A parallel can be made between sap beetles that colonize, develop on, and emerge from oak wilt mats (or wounds) and bark beetles that undergo similar activities in phloem in tree stems and branches. In the cases of both *C. truncatus* and *I. paraconfusus*, the sex ratios of the arriving insects (simulated by response to baited traps) do not appear to match the sex ratios of those that emerge from the developmental substrate. For example, the sex ratio of *I. paraconfusus* responding to the immediate source of naturally produced or synthetic pheromones in

various field studies ranges from 1.8 to 5.6:1 (Struble and Hall, 1955; Gara, 1963; Wood et al., 1967, 1968; Byers, 1983), whereas the sex ratio of insects emerging from ponderosa pine logs has been reported as approximately 1.2:1 (Gara, 1963; Cameron and Borden, 1967) and 1.3:1 (Struble and Hall, 1955). Similarly, with *C. truncatus* we have an arriving sex ratio of 1.8:1 and a sex ratio of 1.1:1 in the colonized oak wilt mats. Further investigation is needed to determine the sex ratio of *C. truncatus* that develop and emerge from oak wilt mats and wounds in oaks. However, higher differential mortality of males during dispersal has been offered as a potential explanation for this sex ratio difference in *I. paraconfusus* (Cameron and Borden, 1967), and this hypothesis may apply to the sex ratio difference for *C. truncatus* as well.

Finally, a number of conclusions with practical implications for monitoring the flight of *C. truncatus* and for managing overland spread of oak wilt can be drawn from this work. Experiments I and II demonstrated that the most attractive bait for *C. truncatus* is the high release rate of pheromone compounds with the bread dough coattractant. Further, experiment V showed that it is unnecessary to add synthetic **2** to the pheromone baits for monitoring *C. truncatus*. Thus, the simplest effective approach to preparation of pheromone baits would be to use synthetic **3**, without bothering to remove the **1** that is invariably present as a synthetic artifact. Since the two- and three-component blends of the *C. truncatus* aggregation pheromone are equally attractive, the less expensive two-component blend can be used effectively as a monitoring tool. As we recorded no response of *C. truncatus* or other sap beetles to 300-ml aliquots of oak chips (experiment VI), operational studies in the future might explore whether landowners, resource managers, or arborists could chip woody debris from uninfected oak trees and leave the chips on site without attracting nitidulid oak wilt vectors to the general area and accelerating overland transmission of the disease. Finally, land managers should be aware that the critical period of flight activity for *C. truncatus* in Minnesota occurs conservatively between early April and early July, and in order to limit the overland spread of oak wilt, pruning and other management activities that wound oaks should be avoided during this time. In a future study, a similar critical period needs to be defined for the other primary oak wilt vector, *Carpophilus sayi*.

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PURINE METABOLIZING CAPABILITY OF *Enterobacter agglomerans* AFFECTS VOLATILES PRODUCTION AND ATTRACTIVENESS TO MEXICAN FRUIT FLY

DAVID C. ROBACKER[1,*] and CAROL R. LAUZON[2]

[1]*Crop Quality and Fruit Insects Research, ARS, USDA*
2413 E. Highway 83, Building 200
Weslaco, Texas 78596, USA

[2]*Department of Biological Sciences*
California State University, Hayward
Hayward, California 94542, USA

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Abstract—We investigated two strains of *Enterobacter agglomerans* that differ in their ability to metabolize uric acid for (1) attractiveness to sugar-fed Mexican fruit flies, and (2) production of volatile chemicals that may be responsible for the attractiveness. The two strains were cultured on a medium that contained uric acid as the primary nitrogen source to simulate bird feces, a natural substrate for this bacterium. Active cultures of both strains were more attractive than uninoculated uric acid medium to both sexes of sugar-fed flies in wind-tunnel bioassays. The uricase(+) strain was more attractive than the uricase(−) strain to males and to females <9 days old, but not to older females. Volatiles found by solid-phase microextraction in greater amounts in headspace above active cultures of both strains than above uninoculated medium were ammonia, dimethyldisulfide, 3-methylbutanol, 2-phenylethanol, 2,5-dimethylpyrazine, and trimethylpyrazine. The uricase(+) strain produced more ammonia, dimethyldisulfide, and trimethylpyrazine than the uricase(−) strain. An additional chemical, 3-hydroxybutanone, appears to be produced exclusively by the uricase(+) strain. The uricase(−) strain produced more 2-phenylethanol than the uricase(+) strain. Differences in volatiles are consistent with the generally greater attractiveness of the uricase(+) strain compared with the uricase(−) strain as ammonia, 3-hydroxybutanone, and trimethylpyrazine have been demonstrated attractive to sugar-fed Mexican fruit flies.

Key Words—Attractants, Diptera, Tephritidae, *Anastrepha ludens*, ammonia, pyrazines, 3-methylbutanol, 3-hydroxybutanone, solid-phase microextraction.

* To whom correspondence should be addressed. E-mail: drobacker@weslaco.ars.usda.gov

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INTRODUCTION

Bird droppings are a well known natural food for many species of tephritid fruit flies (Christenson and Foote, 1960; Malavasi et al., 1983; Hendrichs and Hendrichs, 1990; Hendrichs and Prokopy, 1990). Experiments in which egg production by fruit flies was enhanced when bird feces were added to nitrogen-deficient diets indicated that bird feces may be an important natural source of organic nitrogen to fruit flies (Christenson and Foote, 1960; Hendrichs et al. 1993a,b). However, the principal organic nitrogen in bird feces is uric acid, and fruit flies are unable to metabolize uric acid without assistance from symbiotic microbes (Hendrichs et al., 1993b). Therefore, either the flies themselves or the bird feces must contain the necessary symbionts. Further, it has been shown that the odor of bird feces is attractive to various fruit flies (Prokopy et al., 1992, 1993; Epsky et al., 1997; Robacker et al., 2000). It was demonstrated by addition of antibiotic that bacterial utilization of the feces is largely responsible for their attractiveness to the apple maggot fly (*Rhagoletis pomonella* Walsh) (Prokopy et al., 1993).

In an effort to tie these studies together, Lauzon et al. (1998, 2000) investigated whether the bacteria responsible for making bird feces attractive also serve a role as symbionts that help fruit flies utilize nitrogen from uric acid in the bird feces. MacCollom et al. (1992, 1994) previously had demonstrated that *E. agglomerans* isolates from the apple maggot fly and from its environment were attractive to adult apple maggot flies. Lauzon et al. (1998) isolated several species of bacteria from bird feces and apple foliage and found that two species, *Enterobacter agglomerans* and an atypical strain of *Escherichia coli*, were highly attractive to the apple maggot fly, while other species were not attractive. Further investigation revealed that some strains of *E. agglomerans* isolated from bird feces were not attractive to the flies. These results indicated intraspecific variation in strains of *E. agglomerans* that manifested as phenotypes that were either attractive or nonattractive to the apple maggot fly. Closer examination revealed that the strains differed in their abilities to metabolize various purines and purine degradation products including uric acid, and that this ability was positively related to their attractiveness (Lauzon et al., 2000). Gel electrophoresis indicated that the purine metabolizing strains produced a protein that was characterized as a uricase. Further, they showed that the uricase was synthesized constitutively by the bacteria, i.e., in the absence of uric acid, and that additional uricase production could be induced by uric acid.

As discussed above, the *E. agglomerans* strains that were attractive or nonattractive to the apple maggot fly later proved to be uricase(+) or uricase(−), respectively. Attractiveness to the Caribbean fruit fly (*Anastrepha suspensa* Loew) of one of these *E. agglomerans* strains [later determined uricase(+) by C.R.L.] was demonstrated, ammonia and 3-methylbutanol were identified in volatiles produced by the bacteria, and both chemicals were demonstrated attractive to the flies (Epsky et al., 1998). However, no data were reported for a uricase(−) strain in that study.

Lauzon et al. (1998) suggested identification of volatiles produced by attractive and nonattractive strains of *E. agglomerans*. Such research should further understanding of metabolic differences between strains as well as help to determine which volatiles produced by bacteria are most important for attraction of fruit flies. The research described in this paper was conducted to determine if uricase(+) and uricase(−) strains of *E. agglomerans* differ in attractiveness to the Mexican fruit fly, *Anastrepha ludens* Loew, and if differences in attractiveness can be traced to differences in volatiles produced by the strains. Volatile collection from headspace above bacteria cultures was done with solid-phase microextraction (SPME), a technique used successfully to identify attractive principals produced by numerous bacteria (Robacker and Flath, 1995; Robacker and Bartelt, 1997; Robacker et al., 1998, 2000). Our results showed that both *E. agglomerans* strains are attractive to the Mexican fruit fly, that the uricase(+) strain is more attractive than the uricase(−) strain, that volatiles produced by the two strains differ both qualitatively and quantitatively, and that these differences in volatiles are consistent with observed differences in attractiveness.

METHODS AND MATERIALS

Insects and Test Conditions. Flies were from a culture that originated from yellow chapote fruit (*Sargentia greggii* Coult.), a native citrus host of the fly, collected in Nuevo Leon, Mexico, in 1997. Mixed-sex groups of 20–25 flies (overall sex ratio was 1:1 but individual cups varied) were kept in 473-ml cardboard cartons with screen tops until used in tests. Cups were provisioned with separate sugar and water sources but no protein. Laboratory conditions for holding and testing flies were $22 \pm 2^{\circ}$C, $50 \pm 20\%$ relative humidity, and photophase from 06:30 to 19:30 hr. All tests were conducted between 09:00 and 16:00 hr with 5- to 27-day-old flies. Previous work demonstrated that attraction of Mexican fruit flies to odors of aqueous bacterial cultures was maximum for sugar-fed, protein-deprived flies compared with other feeding regimens, and uniform between 08:30 and 16:30 hr under these temperature and relative humidity conditions for flies between 5 and 15 days posteclosion (Robacker and Garcia, 1993). However, responsiveness decreased by about 50% as flies aged from 15 to 30 days.

Bacterial Cultures. In preliminary work, the two strains of *Enterobacter agglomerans*, designated as uricase(+) and uricase(−), were inoculated individually into tryptic soy broth (Difco Laboratories, Detroit, Michigan, USA). The uricase(+) strain contained a protein characterized as a uricase (Lauzon et al., 2000) that was not present in the other strain. The tryptic soy broth cultures were held under identical conditions, and their growth was monitored and compared over a 24-hr period. Both strains were found to have similar growth rates and were kept under the same growth conditions for all subsequent work.

To prepare bacterial cells for plating, the two strains were grown individually in tryptic soy broth for 18 hr at 28°C. Cells were centrifuged for 20 min at 5000 rpm at room temperature. The supernatant was expelled, and the remaining pellet of cells received approximately 2 ml of sterile distilled water and was vortexed gently to disrupt the pellet. Each cell suspension received additional sterile water and was centrifuged as described earlier. The supernatant was discarded, and this washing procedure was repeated two more times. The final pellet of cells for each strain was adjusted by addition of sterile water to an optical density of approximately 0.550 at 550 nm using a UV-VIS spectrophotometer (Shimadzu Scientific Instruments, Inc., Kyoto, Japan).

A growth medium was prepared that contained uric acid as its primary nitrogen source. Medium was made by combining the following ingredients, per 100 ml of distilled water: 1.6 g phenol red broth (BBL, Becton Dickinson Co., Cockeysville, Maryland, USA); 0.1 g glucose (Sigma Chemical Co., St. Louis, Missouri, USA); 0.1 g dipotasium hydrogen phosphate (J. T. Baker, Phillipsburg, New Jersey, USA); and 1.8 g grade A agar (BBL). This mixture was autoclaved at 121°C at 15 psi for 15 min. The sterilized medium was tempered to approximately 50°C and poured into sterile Petri dishes (plates) containing 1.2 g uric acid (Sigma). Each plate was swirled gently to distribute the uric acid evenly within the medium. The resulting uric acid medium was allowed to solidify, then plates were individually inoculated with 0.1 ml of one of the bacteria strain cell preparations using the spread plate technique. Inoculated and uninoculated plates were sent via overnight delivery service to the USDA-ARS laboratory in Weslaco, Texas, USA.

Wind-Tunnel Bioassay. Bioassays were conducted in a Plexiglas wind tunnel with the dimensions of $0.3 \times 0.3 \times 1.2$ m. Each end of the wind tunnel was screened to allow airflow. The downwind end contained a baffle system to create a uniform airflow through the chamber. Air was pulled through the chamber at 0.4 m/sec by an exhaust fan connected to the downwind end. The top of the chamber had two circular openings (12.8 cm diam.) with Plexiglas covers, located at each end of the chamber, to allow easy access to the chamber's interior. A 75-W "soft white" light bulb (General Electric Co., Cleveland, Ohio, USA) in a reflecting lamp was positioned 17 cm above the downwind end of the chamber. The purpose of this light was to minimize random flying into the upwind end of the chamber by using the flies' positive phototactic reaction. Overhead lighting was provided by two banks of four fluorescent "cool white" lights each (F40CW, General Electric).

Attractiveness of plates containing either active cultures or uninoculated medium was assessed. Plates had been incubated for 2–9 days at 30–32°C prior to testing. Most assays were conducted using incubations of 6–9 days because observations indicated that uric acid utilization was maximum during this period (judged by disappearance of uric acid crystals in medium). To conduct a trial, a plate bottom containing the active culture or uninoculated medium was placed on the floor of the chamber below the opening at the upwind end. One cup of flies was

placed under the downwind opening. Flies were given 5 min to leave the cup, fly or walk upwind, and contact the plate. Upwind movement was scored if flies passed a point two thirds of the distance from the release cup to the plate. An exhaust fan was run for 5 min after each trial to refresh the air in the bioassay room.

Quantification of Volatiles from Cultures. Volatiles in the headspace above active bacterial cultures and above uninoculated medium were quantified by gas chromatography (GC). Plates used in these analyses were selected from every batch of inoculations that were used for bioassays. Incubation times ranged from 2 to 9 days at 30–32°C. Volatiles were sampled using SPME with a polydimethylsiloxane (PDMS) -coated fiber (100 μm coating) (Supelco, Inc., Bellefonte, Pennsylvania, USA). The fiber was inserted into the headspace through a small hole drilled into the side of each plate just prior to volatiles collection. The hole was only slightly larger than the fiber sheath to minimize escape of volatiles from the plate. Sampling time was 1 hr at 30–32°C. On-column injection of volatiles was by thermal desorption from the SPME fiber at 220°C in a 10-cm retention gap (0.53 mm ID deactivated fused silica) connected to the analytical column by a GlasSeal connector (Supelco). The analytical column was a DB-1 (60 m $\times$ 0.32 mm ID, 5-μm film thickness) (J & W Scientific, Folsom, California, USA). For most analyses, column oven temperature was 100°C for 10 min then programmed at 5°C/min to 210°C. However, some analyses were conducted beginning at 50°C after we discovered that 3-methylbutanol and dimethyldisulfide were coeluting in analyses that began at 100°C. No uninoculated controls were conducted for analyses that began at 50°C. Carrier gas was helium at a linear velocity of 30 cm/sec. Analyses were conducted using a Shimadzu GC-17A (Shimadzu Scientific Instruments, Inc., Columbia, Maryland, USA) equipped with both flame ionization (FID) and flame thermionic (model FTD-17) detectors. Detection was by FID for most plates, to analyze ammonia and organic chemicals that did not contain nitrogen. Detection was by FTD for others, to analyze organic chemicals containing nitrogen. GC peak areas were measured using Millennium 2010 Chromatography Manager software (Waters Corporation, Milford, Massachusetts, USA).

Identification of Volatiles Produced by Bacteria. Volatiles produced by bacteria were identified by gas chromatography–mass spectrometry (GC-MS). Two volatile collection methods were used. The first method was to collect volatiles in the headspace above cell suspensions in water. Bacterial cells were scraped from the surface of the medium from one plate each of uricase(+) and uricase(−) strains with a metal spatula and rinsed from the spatula into 1 ml of reverse-osmosis water. The two plates were from the same batch and were incubated for 8 days prior to cell collection. Except during transit to Illinois for GC-MS analysis ($<$24 hr) and during volatiles sampling, cell scrapings were kept at −20°C. Volatiles were collected by SPME with a PDMS fiber (100-μm coating). The fiber was inserted through a septum into the headspace above 0.5 ml of cell suspension in a 2-ml vial for 30 min at room temperature. NaCl and NaOH were added to the vials

to increase the headspace concentrations of organic compounds and basic compounds, respectively (Robacker and Bartelt, 1997).

The second method of volatiles collection was conducted when we discovered that a peak routinely observed by GC-FID analysis of plates containing active cultures was not present in vials with scraped cells. The method described above in which volatiles were sampled directly from plates was then used for GC-MS analysis of one plate each of the uricase(+) and uricase(−) strains incubated for eight days.

Thermal desorption from the PDMS fiber was conducted as described above. The analytical column for GC-MS was a DB-1 fused-silica capillary column (30 m × 0.32 mm ID, 5-μm film thickness). Linear velocity of helium carrier gas was 60 cm/sec. Column oven temperature was 50°C for 1 min, then programmed at 10°C/min to 200°C. GC-MS data were acquired using a Hewlett Packard 6890 GC (Hewlett-Packard Company, San Fernando, California, USA) with an HP 5973 mass selective detector (electron energy = 70 eV) over a mass range of 20–300 amu, at 2 scans/sec. GC-MS identifications were based on computer matching of unknown spectra with those in the Wiley 275K Mass Spectral Database (John Wiley & Sons, New York).

Chemical Attractiveness Bioassay. Cage-top bioassays as described in Robacker and Flath (1995) were used to test attractiveness of two chemicals identified in bacterial volatiles, 3-hydroxybutanone and 2-phenylethanol. The chemicals (both >99%) were obtained from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin, USA). Four quantities of each chemical were tested: 100 ng and 1, 10, and 100 μg. 3-Hydroxybutanone and 2-phenylethanol were prepared in water and methanol, respectively. The bioassay was conducted by placing two filter paper triangles (3 cm/side) containing 10 μl of test chemical solution and two papers containing 10 μl of appropriate solvent (water or methanol) near the corners on top of an aluminum-screened cage (30 cm/side) containing 180–200 adult flies. The papers containing test chemicals were positioned diagonally from each other on the cage top as were the papers containing solvent. The papers were raised 5 mm above the cage top using plastic rings to ensure that olfaction was solely responsible for responses. The number of flies beneath each paper was counted once each minute for 10 min following application of test chemicals. Bioassays were conducted using flies of two feeding regimes. First, the four concentrations of each chemical were bioassayed in random order using flies that were sugar-fed and protein-starved from eclosion. Sugar cubes were removed just prior to bioassays. In most cases, sugar cubes were not replaced, and the same cages were used as sugar-starved flies for bioassays of the same chemical solutions two days later. Each concentration of each chemical was assayed 10–18 times with both sugar-fed and sugar-starved flies.

Statistical Analyses. Attractiveness of the two strains of bacteria in wind-tunnel bioassays was analyzed using SuperANOVA (Abacus Concepts, 1989). For

analysis, proportions of flies to move upwind or contact the plates in each bioassay were transformed by arcsin of the square root because means were highly correlated with variances (Snedecor and Cochran, 1967). Proportions of 0 were replaced with $1/4n$. Separate analyses were conducted for males and females of different age groupings. Effect of bacteria strain on production of volatiles was also tested by analysis of variance of GC area counts using SuperANOVA. Separate analyses were conducted for each chemical. Means separations were done by Fisher's protected least significant difference method (LSD) in all ANOVA's. Paired *t* tests were used to analyze results of cage-top bioassays evaluating attractiveness of individual chemicals. For paired *t* tests, the total of 10 counts of flies at filter papers containing test chemicals were compared with the total at papers containing solvent for each bioassay.

RESULTS AND DISCUSSION

Attractiveness of Uricase(+) and Uricase(−) Strains. More females of ages 5–9 days posteclosion moved upwind toward plates containing cultures of either strain of *E. agglomerans* than plates containing uninoculated medium, and more moved upwind toward the uricase(+) strain than the uricase(−) strain ($F = 13.0$; $df = 2,62$; $P < 0.001$) (Table 1). More of these females also contacted plates containing the uricase(+) strain than uninoculated medium ($F = 3.8$; $df = 2, 62$; $P < 0.05$). More females of ages 10–27 days posteclosion moved upwind toward ($F = 7.0$; $df = 2,67$; $P < 0.01$) and contacted ($F = 5.0$; $df = 2,67$; $P < 0.01$) plates containing cultures of either strain than plates containing uninoculated medium. The number of these females to move upwind toward or contact plates

TABLE 1. ATTRACTION OF SUGAR-FED MEXICAN FRUIT FLIES TO PETRI PLATES CONTAINING TWO STRAINS OF *E. agglomerans* IN A WIND TUNNEL[a]

Fly group	Bacteria strain	Trials *N*	Upwind movement	Contact with plate
Females	Uninoculated medium	16	3.5 ± 1.8 a	1.1 ± 0.8 a
5–9 days old	Uricase(−)	26	14.9 ± 2.7 b	4.6 ± 1.5 ab
	Uricase(+)	26	21.9 ± 2.7 c	8.1 ± 2.1 b
Females	Uninoculated medium	16	3.1 ± 1.2 a	0.4 ± 0.4 a
10–27 days old	Uricase(−)	29	12.8 ± 2.9 b	5.0 ± 1.4 b
	Uricase(+)	29	13.6 ± 3.1 b	7.2 ± 2.6 b
Males	Uninoculated medium	32	3.9 ± 1.0 a	1.1 ± 0.7 a
	Uricase(−)	55	7.7 ± 1.2 b	2.1 ± 0.6 a
	Uricase(+)	55	14.1 ± 1.8 c	5.3 ± 1.2 b

[a] Values are mean percentages (± standard errors) responding out of the total males in the trial. Means in the same fly group and same column followed by different letters are significantly different at the 5% level by Fisher's protected LSD.

containing the uricase(+) strain was not significantly different from the number responding to plates containing the uricase(−) strain. These analyses do not prove that females of the two age groups are significantly different from each other. No actual comparison of the age groups was possible because the experiment was not designed for this test.

Data for males of different ages were combined because analyses indicated that age (5–9 days vs. 10–27 days posteclosion) did not significantly affect responses of males to the *E. agglomerans* cultures. More males moved upwind toward plates containing either strain of *E. agglomerans* than plates containing uninoculated medium and more moved upwind toward the uricase(+) strain than the uricase(−) strain ($F = 13.3; df = 2,132; P < 0.001$; Table 1). More males also contacted plates containing the uricase(+) strain than either uninoculated medium or the uricase(−) strain ($F = 6.0; df = 2,132; P < 0.01$).

Because a wide range of incubation times were used in this work, we examined effects of incubation time on wind-tunnel bioassay results. We could not determine if incubation time affected overall attractiveness of the cultures because incubation time was confounded with time of year when tests were conducted and with batch of flies tested. Effect of incubation time on attractiveness of the two strains relative to each other was not confounded because each strain was tested equally with each fly batch, at all times of the year, and with all incubation times. Partitioning the data in Table 1 by incubation time (2- to 5-day vs. 6- to 9-day incubations), uricase(+) plates were more attractive than uricase (−) plates to younger females and to males of all ages, and uricase(+) and -(−) plates were about equally attractive to older females, regardless of incubation time. Thus, no effects of incubation time on relative attractiveness of the two strains were observed.

These results show that both strains of *E. agglomerans* were attractive to female and male Mexican fruit flies. They also show that the uricase(+) strain was more attractive than the uricase(−) strain, except to mature females. The results differed from those reported for *R. pomonella*, in which isolates of *E. agglomerans* that were found to be uricase(−) in later research (Lauzon et al., 2000) were not more attractive than uninoculated controls (Lauzon et al., 1998). No differences in response with sex or age were reported in studies with *R. pomonella*.

Chemical Identifications and Quantifications. GC-FID and GC-FTD analyses of headspace above active cultures in plates revealed about 100 chemicals in the headspace. It was decided that only peaks with areas of at least 1 mV (about 50 peaks), peaks found in at least three samplings, and peaks found in significantly greater amounts in at least one of the two strains than in the uninoculated controls, would be considered for further analysis. Two exceptions were ammonia and dimethyldisulfide, both of which had mean peak areas below 1 mV. Ammonia was analyzed because it is known to be attractive, and neither FID nor FTD are sensitive to it (it is not carbon based) such that peaks less than 1 mV represent high concentrations. For example, comparable peak sizes were obtained for ammonia and

TABLE 2. GAS CHROMATOGRAPHIC PEAK AREAS OF CHEMICALS IN HEADSPACE OF PETRI PLATE CULTURES OF TWO STRAINS OF *E. agglomerans* SAMPLED BY SOLID-PHASE MICROEXTRACTION[a]

Chemical	Peak area [mV (mean ± SE)]		
	Uninoculated medium	Uricase(−) strain	Uricase(+) strain
Ammonia[b]	0.06 ± 0.02 (8) a	0.13 ± 0.02 (12) b	0.20 ± 0.03 (11) c
Dimethyldisulfide[b]	0.00 ± 0.00 (8) a	0.53 ± 0.11 (8) b	0.96 ± 0.10 (7) c
3-Hydroxybutanone[b]	0.071 ± 0.05 (8) a	0.10 ± 0.10 (16) a	4.3 ± 1.1 (16) b
3-Methylbutanol[b]	0.00 ± 0.00 (8) a	39 ± 7.0 (8) b	27 ± 4.4 (8) b
2-Phenylethanol[b]	0.00 ± 0.00 (8) a	100 ± 14 (16) c	12 ± 2.9 (16) b
2,5-Dimethylpyrazine[c]	190 ± 55 (8) a	310 ± 71 (9) b	400 ± 110 (8) b
Trimethylpyrazine[c]	12 ± 3.3 (8) a	18 ± 4.0 (9) b	32 ± 8.9 (8) c

[a] Number of replications in parentheses; for each chemical, peak areas followed by the same letter were not significantly different from each other by LSD ($P < 0.05$).
[b] Peak areas by FID.
[c] Peak areas by FTD.

2-ethylhexanol despite actual concentrations of 1.8 mg/ml and 30 ng/ml, respectively, in a suspension of duck feces (Robacker et al., 2000). Dimethyldisulfide was analyzed because its GC peak overlapped with that of 3-methylbutanol in initial analyses.

GC-FID and GC-FTD quantifications of ammonia, dimethyldisulfide, and the other peaks remaining under consideration are shown in Table 2. Although actual headspace concentrations of the chemicals were not determined, concentrations of chemicals determined from comparable peak sizes in previous studies of volatiles produced by bacteria were in the range known to be attractive to the Mexican fruit fly (Robacker and Flath, 1995; Robacker and Bartelt, 1997; Robacker et al., 2000).

Both bacteria strains produced more ammonia, dimethyldisulfide, 3-methylbutanol, 2-phenylethanol, 2,5-dimethylpyrazine, and trimethylpyrazine than uninoculated controls (Table 2). The uricase(+) strain produced more ammonia, dimethyldisulfide, 3-hydroxybutanone, and trimethylpyrazine than the uricase(−) strain. In fact, the low amounts of 3-hydroxybutanone found in determinations for the uricase(−) strain and uninoculated medium may represent carryover from previous samplings of the uricase(+) strain, suggesting that 3-hydroxybutanone is not produced by the uricase(−) strain. The uricase(−) strain produced a 2-phenylethanol peak 8 × bigger than that produced by the uricase(+) strain. All of theses chemicals except 3-hydroxybutanone had been identified previously from bacterial odors (Gow, 1954; Drew and Fay, 1988; Lee et al., 1995; Robacker and Flath, 1995; DeMilo et al., 1996; Robacker and Bartelt, 1997; Epsky et al., 1998; Robacker et al., 1998). 3-Hydroxybutanone had been identified previously from yeasts and is known to be attractive to the sap beetle *Carpophilus humeralis* (F) (Coleoptera: Nitidulidae) (Phelan and Lin, 1991; Nout and Bartelt, 1998).

As with attractiveness experiments, data in Table 2 were partitioned to compare emissions of the various chemicals for incubations of 2–5 and 6–9 days. Patterns and amounts of emissions did not vary appreciably by incubation time within this range. Epsky et al. (1997) showed that more ammonia was produced by bird feces incubated for one day compared with longer incubations. Unfortunately, neither our current work nor Epsky et al. (1998) investigated volatiles produced by *E. agglomerans* incubated for shorter periods than two days.

As discussed in the introduction, Epsky et al. (1998) found 3-methylbutanol and ammonia in headspace above tryptic soy agar cultures of a uricase(+) strain of *E. agglomerans*. They reported that 3-methylbutanol and ammonia were the two most abundant volatiles in the headspace and specifically that 3-methylbutanol accounted for >85% of the volatiles detected by GC-MS (ammonia was not analyzed by GC-MS). Although 3-methylbutanol was not the largest peak we observed (Table 2), previous work showed that detection of this chemical in tryptic soy broth cultures of two bacteria species by SPME (Robacker and Bartelt 1997) was much less efficient than by adsorption onto charcoal (Lee et al., 1995; DeMilo et al., 1996), a method similar to that used by Epsky et al. (1998). It also is possible that cultures of the uricase(+) strain in tryptic soy agar may produce more 3-methylbutanol than cultures in uric acid medium. Thus, we do not dispute the statement by Epsky et al. (1998) concerning the abundance of 3-methylbutanol. These authors also speculated that 3-methylbutanol may be an important attractant produced by the uricase(+) strain of *E. agglomerans*, although they did not report volatiles produced by a uricase(−) strain. We have no evidence to refute that 3-methylbutanol may contribute to attractiveness of the uricase(+) strain under some conditions or with some species of fruit flies. However, in our work, 3-methylbutanol cannot account for the greater attractiveness of the uricase(+) strain because the uricase(−) strain produced just as much of this chemical (Table 2).

Bioassays of 3-Hydroxybutanone and 2-Phenylethanol. Attractiveness of 3-hydroxybutanone and 2-phenylethanol in cage-top bioassays was significant by paired *t* tests ($P < 0.05$) for several test quantities evaluated with several groups of sugar-fed and sugar-starved males and females as follows: 3-hydroxybutanone—fed males at the 1 μg test quantity, fed females at 0.1 and 100 μg, and starved females at 100 μg; and 2-phenylethanol—fed males at 10 μg, and starved males and starved females at all test quantities. No dose–response relationships were evident, so data were combined over test quantities, then partitioned into separate analyses for flies <10 days posteclosion versus flies ≥10 days posteclosion.

Results of the age analyses are shown in Table 3. 3-Hydroxybutanone was significantly attractive to 3- to 9-day-old flies including fed males, fed females, and starved males. This is the first report of attractiveness to sugar-fed Mexican fruit flies of a nonpheromonal chemical that does not contain protonizable nitrogen. 3-Hydroxybutanone was not significantly attractive to flies aged 10–14 days

TABLE 3. COUNTS OF SUGAR-FED OR -STARVED MEXICAN FRUIT FLIES OF TWO AGE GROUPS AT SOLVENT BLANKS (S) OR TEST CHEMICALS (T) IDENTIFIED FROM HEADSPACE ABOVE CULTURES OF *E. agglomerans*, USING CAGE-TOP BIOASSAYS

	Counts (mean ± SE)					
	3–9 days old			10–14 days old		
	Trials (*N*)	T	S	Trials (*N*)	T	S
3-Hydroxybutanone						
Fed males	40	38.2 ± 4.4^a	31.2 ± 3.1	20	32.8 ± 4.4	34.4 ± 3.8
Fed females	40	34.3 ± 3.5^a	26.4 ± 2.0	20	28.6 ± 2.5	27.5 ± 1.9
Starved males	16	37.8 ± 4.5^a	30.8 ± 3.3	24	48.7 ± 4.6	45.5 ± 3.7
Starved females	16	26.9 ± 3.8	23.0 ± 2.5	24	39.2 ± 4.2	32.4 ± 2.3
2-Phenylethanol						
Fed males	28	26.8 ± 3.6	22.1 ± 2.8	16	36.9 ± 3.9	35.5 ± 3.2
Fed females	28	23.4 ± 2.8	20.1 ± 2.0	16	35.0 ± 2.3	32.8 ± 2.3
Starved males	16	47.6 ± 4.6^a	34.2 ± 3.5	24	58.0 ± 4.7^a	40.5 ± 2.7
Starved females	16	38.4 ± 4.3^a	30.5 ± 3.7	24	41.0 ± 3.0^a	31.8 ± 2.7

[a] The mean response to a chemical was significantly different from the response to solvent by paired *t* test ($P < 0.05$).

posteclosion. As discussed for age groups responding to the plates, these analyses do not prove that flies of the two age groups are significantly different from each other because the experiment was not designed for this comparison. 2-Phenylethanol was significantly attractive to starved males and starved females of both age groups. It was not significantly attractive to sugar-fed flies.

Relationship Between Bacterial Volatile Emissions and Strain Attractiveness. Emission of three chemicals in greater amounts by the two strains of *E. agglomerans* than by uninoculated medium is consistent with the greater attractiveness of the bacteria to sugar-fed Mexican fruit flies compared with attractiveness of the uninoculated medium. These chemicals, ammonia, 2,5-dimethylpyrazine, and trimethylpyrazine (Table 2), have been reported attractive to sugar-fed Mexican fruit flies (Robacker and Warfield, 1993; Robacker and Flath, 1995; Robacker and Bartelt, 1997; Robacker et al., 2000). The other three chemicals produced in significantly greater amounts by both strains, dimethyldisulfide, 3-methylbutanol, and 2-phenylethanol, were not attractive in tests with sugar-fed Mexican fruit flies here (Table 3) or previously (Robacker and Flath, 1995; Robacker and Bartelt, 1997). However, Epsky et al. (1998) demonstrated that 3-methylbutanol was attractive to *A. suspensa*. 2-Phenylethanol, while not attractive to sugar-fed Mexican fruit flies, was highly attractive to sugar-starved flies (Table 3). This suggests that if sugar-starved flies had been used to evaluate attractiveness of the two bacteria strains, the uricase(−) strain may have been somewhat more attractive because of the high emissions of 2-phenylethanol. It also is possible that some combination

of dimethyldisulfide, 3-methylbutanol, and 2-phenylethanol with other chemicals in the bacterial odor may enhance attraction even though these chemicals were not attractive to sugar-fed flies by themselves. In that regard, Epsky et al. (1998) showed that a combination of 3-methylbutanol and ammonia was more attractive than either chemical alone to *A. suspensa*.

Emission of four chemicals in greater amounts by the uricase(+) strain than by the uricase(−) strain is consistent with the greater attractiveness of the uricase(+) strain to sugar-fed males and 5- to 9-day-old sugar-fed females (Table 1). Three of these chemicals, ammonia, 2,5-dimethylpyrazine, and trimethylpyrazine (Table 2), are attractive to sugar-fed Mexican fruit flies, as discussed above. The fourth, 3-hydroxybutanone, is attractive to sugar-fed males and 3- to 9-day-old sugar-fed females (Table 3). The lack of attractiveness of 3-hydroxybutanone to sugar-fed females 10 days posteclosion or older (Table 3) is also consistent with the lower attractiveness of the uricase(+) strain to 10- to 27-day-old sugar-fed females (Table 1). However, sugar-fed males 10 days posteclosion or older also were not attracted to 3-hydroxybutanone (Table 3), but no corresponding decrease in attractiveness of the uricase(+) strain to older males occurred in this case.

This is not the first time that production of volatiles and attractiveness to the Mexican fruit fly by different strains of the same bacterium have been investigated. Previously, Robacker et al. (1998) showed that four different strains of *Bacillus thuringiensis* produced widely different amounts of eight volatile chemicals known to be attractive to this fly. However, in that case, aqueous filtrates of the four strains did not differ in attractiveness as each contained enough of several of the chemicals to compensate for lesser amounts of some of the others.

Metabolic Capabilities, Attractiveness, and Symbioses. The medium used to culture *E. agglomerans* in this work was designed to simulate bird feces in that uric acid was the primary nitrogen source. We expected that only the uricase(+) strain would utilize uric acid efficiently. As a consequence, we also expected that the uricase(−) strain would utilize other biochemicals either more or less efficiently than the uricase(+) strain as different enzyme systems were induced to metabolize the usable nutrients. It follows that the greater amounts of ammonia, dimethyldisulfide, 3-hydroxybutanone, 2,5-dimethylpyrazine, and trimethylpyrazine (Table 2) produced by the uricase(+) strain were the result of this strain's metabolic capability on this special medium and may account for its greater attractiveness to *R. pomonella* (Lauzon et al., 1998) and *A. ludens*. Conversely, the greater amount of 2-phenylethanol produced by the uricase(−) strain (Table 2) may reflect this strain's metabolic capability. As discussed above, the uricase(−) strain may possibly be more attractive than the uricase(+) strain to sugar-hungry flies that respond strongly to 2-phenylethanol (Table 3) and less strongly to ammonia and at least some other nitrogen containing compounds (Robacker et al., 2000).

E. agglomerans strains are known to grow on variable and numerous substrates (Lauzon et al., 1998). In part, this is why biochemical identification of this bacterium can be a challenge. Thus, it was not surprising that *E. agglomerans* strains isolated from different sources possessed different metabolic capabilities (Lauzon et al., 2000). Results reported here (Table 2) and elsewhere (Robacker et al., 1998) indicate that different strains of the same bacterium can produce different volatile metabolites even when cultured on the same medium. It also is known that the same strain can produce different volatiles or at least different amounts of the same chemicals when grown on different substrates (Epsky et al., 1998). This was the reason we used a special uric acid medium to test differences between uricase(+) and -(−) strains of *E. agglomerans*. Further, it has been demonstrated that different species or strains of bacteria can produce many of the same chemicals (Robacker et al., 1998). In this case, it is possible that the same compounds could be produced by either identical or different metabolic pathways depending on the precursor available to the bacteria and perhaps other environmental factors. Incubation time or stage in the growth cycle of the bacteria is another factor that can affect utilization of substrate and production of volatile metabolites (Epsky et al., 1997). Thus, the dynamic and plastic nature of bacterial metabolism must be taken into consideration when trying to interpret insect response to bacterial odors. We also discussed that insects may respond differently to bacterial odors depending on their physiological condition related to age (Table 1) and nutritional state (Robacker and Garcia, 1993; Robacker and Moreno, 1995). All of this complicates evaluation of insect attraction to bacterial odors.

Despite nonuniformities in bacterial odor production and fly responses, there is reason to suspect that some bacteria form close symbiotic relationships with insect hosts, an idea that has been bandied about for decades but not proven. The close physical association between *E. agglomerans* and several tephritids, the presence of this bacterium in the natural food of these flies and on their host plants, and attraction by these tephritids to specific strains of the bacterium suggest this bacterium performs an important role in the life cycle of tephritids. The chemical profiles characteristic of the various strains of *E. agglomerans*, coupled with responses by fruit flies of specific physiological states, may give us some idea of the contributions these bacteria make to insect survival and perhaps provide insight to the needs flies have at distinct physiological times in their adult life.

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KAIROMONE STRAINS OF *Euclytia flava* (TOWNSEND), A PARASITOID OF STINK BUGS[1]

J. R. ALDRICH* and A. ZHANG

USDA-ARS Chemicals Affecting Insect Behavior Laboratory
Agricultural Research Center-West
Beltsville, Maryland, USA 20705

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Abstract—Tachinid flies commonly use the pheromones and allomones of stink bugs (Pentatomidae) as host-finding kairomones. Pheromone-baited traps for predaceous (*Podisus* spp.) and phytophagous (*Euschistus* spp.) pentatomids were used to obtain tachinid parasitoids in order to study the semiochemical relationships between these parasitic flies and their stink bug hosts. Gas chromatography–electroantennogram detector (GC-EAD) experiments and field tests were conducted to determine if pheromone strains of the tachinids, *Euclytia flava* and *Hemyda aurata*, occur in nature and to determine if the EAD-active compound, (*E*)-2-octenal (a common allomone compound of Heteroptera), affects attraction of tachinid parasitoids to synthetic *Podisus* pheromones. Addition of (*E*)-2-octenal to *Podisus* spp. synthetic pheromones in field traps tended to suppress attraction of the bugs, whereas (*E*)-2-octenal decreased, did not affect, or increased pheromonal attraction of tachinid parasitoids depending on the host species pheromone being tested and the habitat type in which the traps were deployed. Evidence from GC-EAD experiments of *E. flava* associated with different stink bug hosts suggested that kairomone-strains of this tachinid parasitoid coexist naturally. The significance of cryptic kairomone strains of parasitoids for classical biological control is discussed, and the mechanisms whereby parasitoids evolve kairomonally mediated host-shifts is considered.

Key Words—Pheromone, allomone, *Hemyda*, *Gymnosoma*, hexenal, linalool, α-terpineol, methyl (*E*,*Z*)-2,4-decadienoate, host shift, speciation.

[1] Mention of commercial products does not constitute an endorsement by the USDA.

* To whom correspondence should be addressed. E-mail: aldrichj@ba.ars.usda.gov

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INTRODUCTION

Stink bugs, as the name implies, emit secretions unpleasant to humans and invertebrates alike—from metasternal glands (adults) or dorsal abdominal glands (nymphs)—to defend themselves (Aldrich, 1995b). Pentatomids and other true bugs (Hemiptera: Heteroptera) also produce pheromones from a variety of glandular sources (Aldrich, 1995b; McBrien and Millar, 1999). However, heteropteran chemical defense and communication are not without risks because many parasitoids exploit their allomones and pheromones as host-finding kairomones, especially scelionid wasps (egg parasitoids) and tachinid flies (parasitoids of nymphs and adults) (Mattiacci et al., 1993; Aldrich, 1995b; Bruni et al., 2000). Here we describe our efforts to understand the semiochemical relationships between tachinid fly parasitoids and their predaceous (*Podisus* spp.) and phytophagous (*Euschistus* spp.) pentatomid hosts.

In Maryland, *Podisus maculiventris* (Say) (called the spined soldier bug) is a common bivoltine general predator (McPherson, 1982), while the sympatric species, *P. neglectus* Westwood (= *P. fretus*), is a relatively uncommon univoltine specialist predator of pine sawfly larvae (Hetrick, 1959; Thomas, 1992). These (and other) *Podisus* males have enlarged dorsal abdominal glands from which they selectively release pheromones to attract conspecific females. Conspecific males also go to calling males, probably to find potential mates without advertising themselves to parasitoids, and nymphs walk to the pheromone, perhaps because pheromone emission is associated with prey (Aldrich, 1995b). The attractant pheromones of *P. maculiventris* and *P. neglectus* each contain (*E*)-2-hexenal and benyzl alcohol as major constituents, but the pheromones differ in their principal monoterpenoid component; in *P. maculiventris* α-terpineol is the essential synergist for the other essential synergist, (*E*)-2-hexenal (Aldrich et al., 1984a), whereas in *P. neglectus* linalool replaces α-terpineol as an essential synergist (Aldrich et al., 1986). Adult *Podisus* females possess glands homologous to the hypertrophied male pheromone glands; however, in females these glands are tiny, and produce a unique array of chemicals (Aldrich et al., 1984b). In addition, *Podisus* nymphs produce chemically distinctive exocrine secretions from dorsal abdominal glands (Aldrich, 1995b).

Euschistus is a New World genus of phytophagous stink bugs, several of which are agricultural pests that are all the more important nowadays because of their immunity to *Bt* endotoxins expressed in genetically modified crops (Bacheler, 1998). In North America, the genus *Euschistus* includes several species whose males produce an attractant pheromone (presumably from gland cells scattered in the abdominal epidermis) with one dominant compound, methyl (*E*,*Z*)-2,4-decadienoate (Aldrich et al., 1991b). Synthetic methyl (*E*,*Z*)-2,4-decadienoate attracts females, males, and nymphs of several *Euschistus* spp. Minor pheromone components that may account for species-specificity of *Euschistus* pheromones

have yet to be discovered despite attempts to do so (Aldrich et al., 1995). As in *Podisus* spp., secretions presumed to be allomones are produced in metasternal scent glands (adults) and dorsal abdominal glands (nymphs) of *Euschistus* spp., and the secretions from these glands are chemically similar to those of other stink bugs (Borges and Aldrich, 1992).

Large numbers of one tachinid parasitoid, *Euclytia flava* (Townsend), can be captured alive in traps baited with synthetic pheromones mimicking the male-produced pheromones of either *P. maculiventris* or *P. neglectus* (Aldrich, 1995a). A second tachinid parasitoid, *Hemyda aurata* Robineau-Desvoidy, is commonly attracted to, but will not usually enter, traps baited with these pheromones; however, substantial numbers of this tachinid can be reared from field-collected *Podisus* adults. Female tachinid parasitoids of stink bugs usually lay macro-eggs on adult hosts (McPherson et al., 1982), but late-stage nymphs are also attacked (Buschman and Whitcomb, 1980; Eger and Ables, 1981; Panizzi and Slansky, 1985). Indeed, both *E. flava* and *H. aurata* were significantly attracted to a synthetic blend mimicking the allomone of spined soldier bug nymphs (Aldrich, 1995b). *Euclytia flava* was occasionally collected in traps baited with the main *Euschistus* pheromone component methyl (*E*,*Z*)-2,4-decadienoate (Aldrich et al., 1991b) and, in the laboratory, females of this fly oviposited on a variety of pentatomids, actually preferring to lay on exotic species when given a choice between exotic and native bugs (Aldrich, 1995a). Therefore, we consider *E. flava* to be more of a generalist parasitoid (Oetting and Yonke, 1971; Buschman and Whitcomb, 1980; Jones and McPherson, 1980; Aldrich, 1995a) than is *H. aurata* (Eger and Ables, 1981; McPherson et al., 1982), which we have not encountered in or around traps baited with methyl (*E*,*Z*)-2,4-decadienoate (Aldrich et al., 1991b).

In the experiments described here we sought to explore the electrophysiological sensitivity of tachinid flies to the various exocrine secretions of preferred and nonpreferred hosts. Preliminary gas chromatography–electroantennogram (GC-EAD) experiments, using chilled preparations similar to those for apple maggot flies (Zhang et al., 1999), indicated that preparations of *E. flava* antennae could be maintained for hours and that their antennae were more sensitive to the essential *Podisus* pheromone components than were those of the bugs themselves. Furthermore, *E. flava* antennae were extremely sensitive to the common stink bug defensive compound (Aldrich, 1995b), (*E*)-2-octenal (Figure 1A below). Specific objectives of the research were to determine if kairomone strains of *E. flava* or *H. aurata* occur in nature and to determine if (*E*)-2-octenal affected attraction of tachinid parasitoids to synthetic *Podisus* pheromones.

MATERIALS AND METHODS

Chemical Standards and Treatments. Standards of the following compounds were purchased or prepared: (*E*)-2-hexenal (**1**), (*E*)-2-octenal (**3**), (*E*)-2-nonenal

(**6**), (*E*)-2-decenal (**8**), (*E*,*E*)-2,4-octadienal (**18**), methyl (*E*,*Z*)-2,4-decadienoate (**19**) (Bedoukian Research, Inc., Danbury, Connecticut, USA); hexanal (**10**), 1-hexanol (**11**), hexanoic acid (**15**), (*E*)-2-hexenoic acid (**14**), benzaldehyde (**12**), benzyl alcohol (**2**), *n*-dodecane (**7**), *n*-tridecane (**9**), linalool (**16**) (Aldrich Chemicals, Milwaukee, Wisconsin, USA); α-terpineol (**4**) (Fisher Scientific Company, Fair Lawn, New Jersey, USA); tetradecanal (**17**) (= myristyladehyde) (Fluka Chemika, Buchs, Switzerland). (*E*)-4-Oxo-2-hexenal (**5**) was synthesized according to Ward and VanDorp (1969), and hexyl hexanoate (**13**) was prepared by the standard acid-chloride procedure. Standard solutions for GC-EAD were prepared as follows: (*E*)-2-octenal and (*E*)-2-decenal; α-terpineol, linalool and (*E*)-2-octenal standards; (*E*)-2-octenal, linalool, α-terpineol and methyl (*E*,*Z*)-2,4-decadienoate; and methyl (*E*,*Z*)-2,4-decadienoate alone (10 ng each standard/μl CH_2Cl_2; the same solution was used for all data presented in Table 1 below).

Three-component blends were prepared to mimic the natural pheromones of *P. maculiventris* and *P. neglectus* as follows. (*E*)-2-Hexenal, racemic α-terpineol, and benzyl alcohol were mixed in a molar ratio of 1:0.843:0.061, respectively. Batches of 40 ml of pheromone (17.6 ml hexenal, 21.4 ml α-terpineol, 1 ml benzyl alcohol) were normally prepared. A 20% (v/w) formulation of pheromone in plasticized polyvinyl chloride (PVC) was prepared by mixing 107 g of powdered PVC (Tenneco, Piscataway, New Jersey, USA) with 53 ml of dioctyl phthalate (Aldrich Chem.), then adding the pheromone (Fitzgerald et al., 1973). The pheromone–PVC mixture was poured into test tubes (to the top), heated at 110°C for 30 min, removed from the tubes, and stored in sealed vials in a freezer until use (PM treatment). Pheromone for *P. neglectus* (PN treatment) was prepared in a similar manner except linalool (Aldrich Chem.) was substituted for α-terpineol (Aldrich et al., 1986). (*E*)-2-Octenal was formulated separately by adding 20 ml of the standard to PVC prepared as described above (O treatment). Methyl (*E*,*Z*)-2,4-decadienoate (Bedoukian Res.) inhibits plasticization of PVC; therefore, this treatment was prepared as a 1:2 solution in heptane (E treatment).

Trapping. Traps were made of transparent cylindrical containers having either white or transparent lids (20.2 × 19.7 cm; Pioneer Plastics, Inc., Dixon, Kentucky, USA) by cutting two holes (9 cm diam.) in opposite sides, and covering each hole with an inwardly projecting screen funnel. The containers were turned upside-down, so that the normal top of the container became the trap bottom, small drainage holes were made in the bottom, and the bottoms were held in place by two elastic cords hooked into each screen funnel. Traps were hung from branches about 1.7 m from the ground, at least 25 m apart, along power line cuts or dirt roads at Beltsville Agricultural Research Center-East (BARC-East; 39°2′N/76°55′W). Ten to 20 traps baited with ~350 mg of *P. maculiventris* pheromone-PVC material were deployed in mid-March each spring from 1999 through 2001 in deciduous

forest tracts at the BARC-East to harvest adult spined soldier bugs emerging from overwintering and to collect tachinids. These traps were monitored every one to two days, and rebaited every three to four days through July, after which bugs and tachinids became scarce.

In 1999, an experiment was conducted from May 21 through June 28 to test pheromones of *P. maculiventris* and *P. neglectus*, with and without (*E*)-2-octenal added, using traps deployed in deciduous and coniferous forest tracts at BARC-East (see Aldrich et al., 1986). The protocol for this experiment was similar to that described above. Six traps were deployed per treatment (four treatments: PM, PN, PM+O, PM+O) in each area (48 traps total), alternating treatments. For treatments that included (*E*)-2-octenal, a ~350 mg slice of PVC containing (*E*)-2-octenal was included in the trap with the PM or PN material.

In 2001, as part of another study to test different formulations of methyl (*E*,*Z*)-2,4-decadienoate, 20 traps were baited every two to three days by applying 10 μl of the heptane solution containing this methyl ester to gray rubber septa (5 × 9 mm; Thomas Scientific, Philadelphia, Pennsylvania, USA), plus 10 heptane-baited control traps. These traps were deployed as above at BARC-East and monitored regularly from August 31 through October 15.

Insects. Podisus maculiventris adults caught in pheromone-baited traps were brought to the laboratory and maintained in groups of 10–15 bugs per $^1/_2$-pint ice cream carton provided with mealworm pupae (Rainbow Mealworms, Inc., Compton, California, USA), fresh green beans (Leguminosae: *Phaseolus vulgaris* L.), and water (Aldrich and Cantelo, 2000). To collect *H. aurata* puparia, holes were punched in the bottom of the containers to allow larvae that emerged from bugs to escape predation by crawling through the holes into a Petri dish underneath the carton.

Colonies of *E. servus* and *E. tristigmus* were started from adults collected in May and June, 2000 and 2001, from Empress trees (Scrophulariaceae: *Paulownia tomentosa* (Thunb.) Steud.) at BARC-East and reared as described earlier (Aldrich et al., 1995). Trapped *Euschistus* spp. were also brought to the laboratory and held separately from the laboratory colonies to collect emergent tachinids.

Most *E. flava* used for GC-EAD experiments were collected in or around pheromone-baited traps, but a few individuals of this species were reared from *P. maculiventris* caught in PM- or PM+O-baited traps. One *E. flava* female was collected on October 11, 2001, in a trap baited with methyl (*E*,*Z*)-2,4-decadienoate, and this *Euschistus*-associated fly was used for GC-EAD. For *H. aurata* associated with *P. maculiventris*, approximately equal numbers of flies were reared from wild spined soldier bugs as were collected on or around PM- or PM+O-baited traps. For *P. neglectus*-associated *H. aurata*, all flies used for GC-EAD were collected on or around PN- or PN+O-baited traps. *Gymnosoma* sp. used for GC-EAD were both field-collected and reared from wild *E. tristigmus*.

Field-collected flies were caught in cotton-stoppered shell vials and transported to the laboratory in a cooler. Field-collected and laboratory-reared tachinids were segregated according to their associated host, confined in plastic pint containers provided with water and honey, and held in an insectary until use (16L:8D cycle, ~65% relative humidity, and ~25°C). Regardless of the source of tachinids, care was taken not to anesthetize the flies for fear that this might adversely affect antennal response; instead, flies were briefly chilled prior to killing for GC-EAD experiments.

Volatile Extracts. Dorsal abdominal and metasternal glands (DAGs and MGs, respectively) from *P. maculiventris* adults were excised from CO_2-anesthetized bugs submerged in tap water and extracted in CH_2Cl_2 as follows: 1 pair of DAGs/~300 μl solvent (male), 5 pairs of DAGs/~50 μl solvent (females), and 1 MG/~200 μl solvent (male or female). The contents of the nymphal DAGs are shed with the exuviae, and the secretion extracted from fresh exuviae has been shown to match the secretion obtained from live nymphs (Aldrich et al., 1991a; Borges and Aldrich, 1992); therefore, the fifth-instar exuviae from ~20 *P. maculiventris* nymphs that had molted to adults within 24 hr were collected and extracted in ~500 μl CH_2Cl_2. *Euschistus servus* pheromone extracts were obtained by aeration using Super Q adsorbent (Alltech Associates, Inc., Deerfield, Illinois, USA) as recently described (Borges et al., 2001).

Gas Chromatography–Electroantennogram Detector Analysis. The GC-EAD system used was as previously described (Zhang et al., 1997; Zhang et al., 1999) with some modifications. A Hewlett Packard 6890 gas chromatograph equipped with a 60-m × 0.25-mm-ID, 0.25-μm film-thickness DB-5 or DB-WAXetr capillary column (J&W Scientific Inc., Folsom, California, USA) in the splitless mode with nitrogen as carrier (2 ml/min) was used for GC (50°C for 2 min, then programmed to 230°C at 10°C/min and held for 10 min). Antennal recordings were made using a holder constructed from a piece of acrylic plastic (8 cm long × 0.8 cm wide × 0.6 cm thick) into which two 1.25-mm-diam. wells were drilled near the end of the plastic about 3 mm apart with a notch cut between the two wells to allow airflow. Each well was filled with 0.9% NaCl solution, and gold wires immersed in each saline-filled well electrode were connected to a high-impedance 1:100 amplifier with automatic baseline drift compensation. The EAD preparation was made using the whole head of a fly immersed in one well with the tips of the antennae making contact with the saline in the other well via a short lateral capillary tube inserted in the side of the other well. The antennal preparation was cooled to ~5°C inside a condenser by circulating near 0°C water from a benchtop refrigeration unit (RTE-100, Neslab Instruments, Inc., Portsmouth, New Hampshire, USA). The flame ionization and electrophysiological output signals (effluent split in 1:1 ratio) were recorded using Hewlett-Packard ChemStation software.

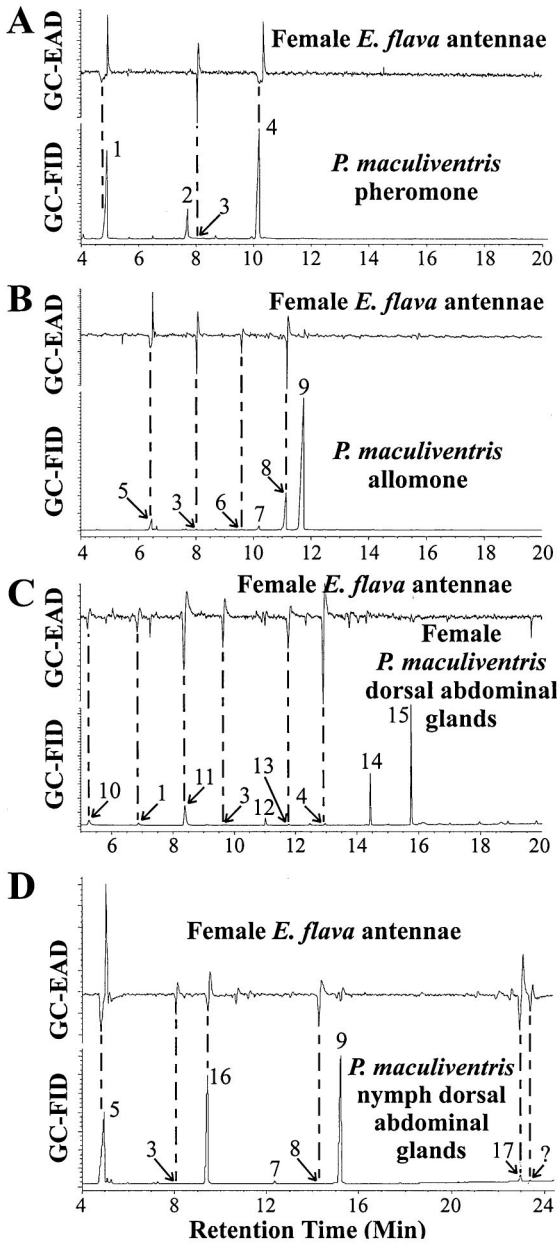
Compound Identification. Samples were analyzed by gas chromatography–mass spectrometry (GC-MS) on an HP 6890 GC coupled to an HP 5973 Mass Selective Detector using the same type of GC column and conditions as listed above except with helium as carrier gas. Identifications based on mass spectrometry were verified by chromatographic comparison to known standards, some of which were also used for GC-EAD experiments

Statistical Analysis. Analysis of variance was performed with the MIXED procedure (SAS Institute Inc., 1997) on the raw data counts. Treatments labeled with the same letter are not significantly different at the 0.05 significance level (Figures 3 and 4 below).

RESULTS

In general, tachinid EAD preparations remained active for four to eight GC runs (~30 min/run) and were extremely repeatable with low background noise after the first or second GC run. In contrast, antennal preparations of *Podisus* and *Euschistus* bugs (not shown) were relatively insensitive and unstable.

Representative GC-EAD runs of *E. flava* female antennal preparations to the array of *P. maculiventris* exocrine gland extracts are shown in Figure 1. The initial GC-EAD experiments showed that this tachinid is not equally sensitive to all components of the male-produced spined soldier bug pheromone (Figure 1A). Responses to the essential components, (*E*)-2-hexenal (**1**) and α-terpineol (**4**), were so extreme that apparent overload occurred as evidenced by the unusual initial deflection of the EAD signal, whereas benzyl alcohol (**2**) elicited almost no antennal response. A strong EAD response was consistently observed to a barely FID-detectable component of the male DAG secretion that was then identified as (*E*)-2-octenal (**3**) (Figure 1A). The *P. maculiventris* adult allomone (Figure 1B) elicited strong *E. flava* antennal responses to (*E*)-4-oxo-2-hexenal (**5**), (*E*)-2-octenal (**3**), (*E*)-2-nonenal (**6**), and (*E*)-2-decenal (**8**), but little or no response was elicited by either dodecane (**7**) or tridecane (**9**). In DAG extracts from *P. maculiventris* females (Figure 1C), the relatively minor components, (*E*)-2-octenal (**3**) and (*E*)-2-decenal (**8**), elicited strong antennal responses in *E. flava* [stronger than that for (*E*)-2-hexenal (**1**)], and a heretofore unknown component of this gland (Aldrich et al., 1984b), hexyl hexanoate (**13**), was strongly EAD-active. The more abundant components, hexanal (**10**) and 1-hexanol (**11**), elicited weak and strong EAD responses, respectively; while *E. flava* antennae were insensitive to the major components, hexanoic (**14**) and (*E*)-2-hexenoic (**15**) acids. Finally, GC-EAD experiments with the *P. maculiventris* exuvial extract (Figure 1D) corroborated the *E. flava* antennal responses to those compounds in common with components of adult exocrine gland secretions and demonstrated that *E. flava* is highly sensitive to linalool (**16**) as well as tetradecanal (**17**). No obvious differences in antennal


A
GC-EAD
GC-FID
Female E. flava antennae
P. maculiventris
pheromone
1
2
3
4
4 6 8 10 12 14 16 18 20
B
GC-EAD
GC-FID
Female E. flava antennae
P. maculiventris
allomone
5
3
6
7
8
9
4 6 8 10 12 14 16 18 20
C
GC-EAD
GC-FID
Female E. flava antennae
Female
P. maculiventris
dorsal abdominal
glands
10
1
11
3
12
13
4
14
15
6 8 10 12 14 16 18 20
D
GC-EAD
GC-FID
Female E. flava antennae
P. maculiventris
nymph dorsal
abdominal
glands
5
3
16
7
8
9
17
?
4 8 12 16 20 24
Retention Time (Min)

responses were observed for antennal preparations of male *E. flava* to these extracts (not shown).

The relative sensitivity of *E. flava* antennae to (*E*)-2-octenal (**3**) versus (*E*)-2-decenal (**8**) was tested by GC-EAD using a 10 ng/μl CH_2Cl_2 solution (Figure 2A). *Euclytia flava* antennae appeared to be more sensitive to (*E*)-2-octenal than (*E*)-2-decenal; therefore, it was decided to use (*E*)-2-octenal in the subsequent field experiment.

The captures of *Podisus* adults in traps baited with pheromone alone or pheromone plus (*E*)-2-octenal are shown in Figure 3. Only *P. maculiventris* adults were caught in PM- or PM+O-baited traps, and only *P. neglectus* were caught in PN-baited traps; there was no crossover attraction between pheromones. Significantly more *P. maculiventris* adults were caught in PM− and PM+O-baited traps in deciduous forest than coniferous forest but, within a given habitat and treatment, the captures of males and females were not significantly different (Figure 3A,B). Addition of (*E*)-2-octenal to the PM treatment tended to decrease the captures of spined soldier bug adults; this suppression was significant for males trapped in the deciduous forest habitat (Figure 3A), and females trapped in coniferous forest (Figure 3B). *Podisus neglectus* was caught solely in coniferous forest, and only about a tenth as many individuals were caught as the number of *P. maculiventris* caught in deciduous forest (Figure 3). Inclusion of (*E*)-2-octenal with the PN-treatment suppressed attraction of *P. neglectus* adults, significantly so for females (Figure 3B).

The responses of *E. flava* adults to the chemical treatments tested in the field are shown in Figure 4. In both deciduous and coniferous forest habitats, significantly more *E. flava* were caught in traps baited with PM or PM+O than in traps baited with PN or PN+O. In addition, more *E. flava* were captured within the deciduous forest site than the coniferous forest site. Addition of (*E*)-2-octenal to the PM treatment tended to reduce attraction of *E. flava* adults in the deciduous forest area (significant for males) (Figure 4A), but this trend was not evident for traps in the coniferous forest area (Figure 4B). Conversely, addition of

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FIG. 1. Gas chromatogram–electroantennogram detector experiments for antennal preparations of *Euclytia flava* females caught in traps baited with *Podisus maculiventris* pheromone to *P. maculiventris* exocrine gland extracts of: (A) male dorsal abdominal gland (attractant pheromone), (B) adult metasternal gland extract (allomone), (C) female dorsal abdominal glands extract, and (D) fifth-instar nymph dorsal abdominal glands (A, B, and D run with DB-5 GC column; C run with DB-WAXetr column; other conditions as described in text; **1** = (*E*)-2-hexenal, **2** = benzyl alcohol, **3** = (*E*)-2-octenal, **4** = α-terpineol, **5** = (*E*)-4-oxo-2-hexenal, **6** = (*E*)-2-nonenal, **7** = dodecane, **8** = (*E*)-2-decenal, **9** = tridecane, **10** = hexanal, **11** = 1-hexanol, **12** = benzaldehyde, **13** = hexyl hexanoate, **14** = hexanoic acid, **15** = (*E*)-2-hexenoic acid, **16** = linalool, and **17** = tetradecanal).

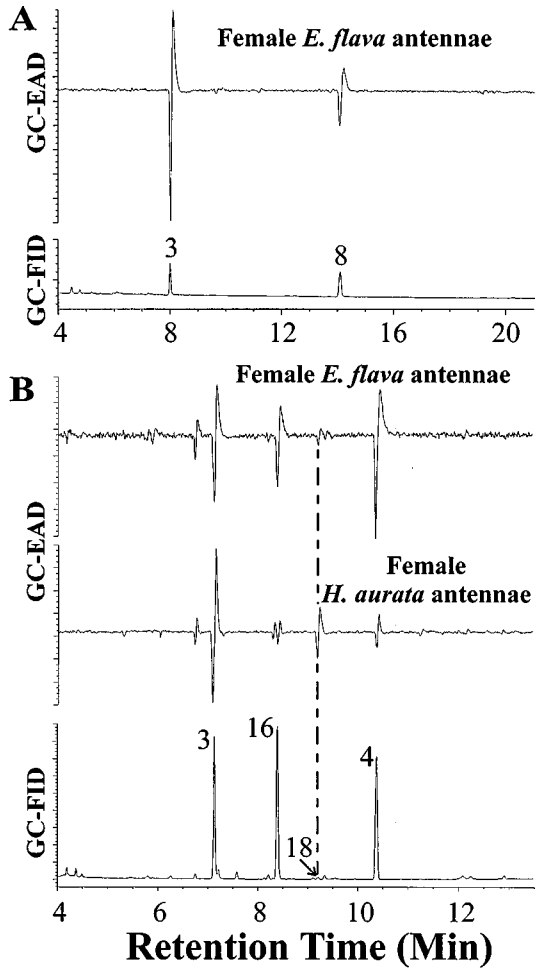


FIG. 2. Gas chromatogram-electroantennogram detector experiments for *Podisus maculiventris*-associated females of (A) *Euclytia flava* antennae to (*E*)-2-octenal (**3**) and (*E*)-2-decenal (**8**) standards (~10 ng each; DB-5 column) and (B) *E. flava* and *Hemyda aurata* antennae to (*E*)-2-octenal (**3**), linalool (**16**) and α-terpineol (**4**) standards (10 ng/μl CH_2Cl_2; ~3 μl/injection; DB-WAXetr column).

(*E*)-2-octenal to the PN treatment tended to increase attraction of *E. flava* females trapped both in the deciduous and coniferous forest sites (Figure 4). Significantly fewer *E. flava* males were attracted to both PN and PN+O treatments in each forest type.

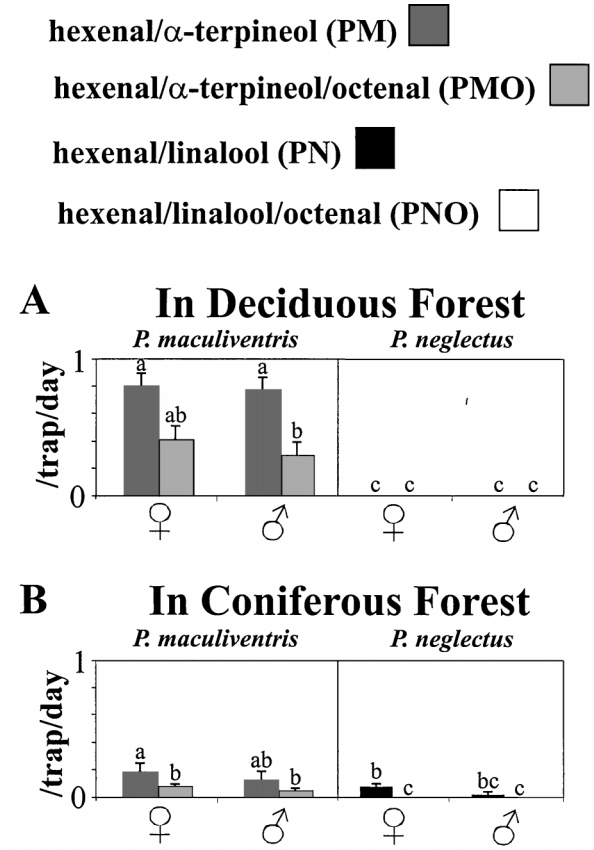


FIG. 3. Attraction of *Podisus* spp. to traps baited with pheromones (PM and PN) and with pheromones plus (*E*)-2-octenal (PMO and PNO) deployed in deciduous and coniferous forest tracts from May 21 through June 28, 1999 (6 traps/treatment/forest type; other details described in text; within a forest type, bars with different letters are significantly different at the 5% level).

Typical EAD traces for *E. flava* and *H. aurata* antennae toward a standard solution of (*E*)-2-octenal (**3**), linalool (**16**), and α-terpineol (**4**) are shown in Figure 2B (no differences noted between female and male flies). In addition to the clear responses to these known host volatiles, other responses were consistently observed to unknown compounds present in the standard solution, one of which was tentatively identified as (*E*,*E*)-2,4-octadienal (**18**).

Table 1 presents the relative antennal responses (calculated as amplitude ratios) of *H. aurata* and *E. flava* associated with particular stink bug species. The

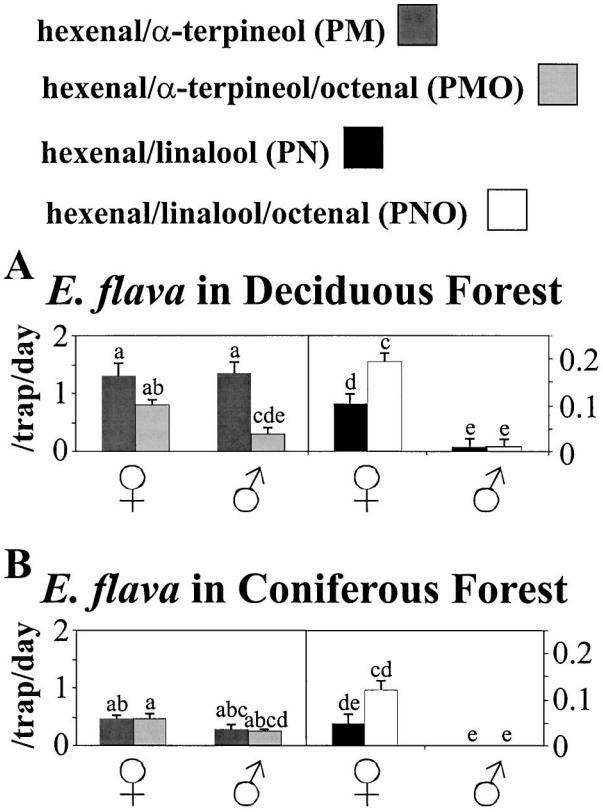


FIG. 4. Attraction of *Euclytia flava* to traps baited with pheromones (PM and PN) and with pheromones plus (*E*)-2-octenal (PMO and PNO) (conditions as for Figure 3).

outstanding difference in EAD-responses between *H. aurata* and *E. flava* is the relatively greater sensitivity of *H. aurata* to (*E*)-2-octenal versus the pheromone monoterpenoids; the (*E*)-2-octenal/α-terpineol ratio for *H. aurata* is >5 times that for *E. flava* from traps baited with *Podisus* pheromones. EAD data for *E. flava* from *P. maculiventris* and *P. neglectus* pheromone-baited traps do not indicate significant differences between these populations of flies; 2.12 ± 0.34 and 1.95 ± 0.13 for α-terpineol/linalool, respectively, and 0.73 ± 0.05 and 0.69 ± 0.05 for (*E*)-2-octenal/α-terpineol, respectively. In contrast, the *E. flava* individual collected in a *Euschistus* pheromone-baited trap was almost totally insensitive to linalool (Figure 5A), yielding an α-terpineol/linalool ratio of 14.91 ± 2.46 (Table 1). However, this fly exhibited sensitivities to α-terpineol and (*E*)-2-octenal comparable to those of *Podisus*-associated *E. flava* as indicated by its (*E*)-2-octenal/α-terpineol

TABLE 1. RATIOS OF ANTENNAL RESPONSES TO KAIROMONE COMPONENTS FOR TACHINIDS, *Euclytia flava* AND *Hemyda aurata*, OBTAINED FROM PHEROMONE-BAITED TRAPS OR BY REARING FROM FIELD-COLLECTED *Podisus maculiventris*

Species	Source[a]	*N* (Replicates)	α-Terpineol/linalool (mean ± SEM)	(*E*)-2-Octenal/α-terpineol (mean ± SEM)
H. aurata	From SSB[b]	6 (17)	1.40 ± 0.08	3.96 ± 0.40
E. flava	PM/PMO traps[b]	19 (42)	2.12 ± 0.34	0.73 ± 0.05
E. flava	PN/PNO traps[b]	7 (17)	1.95 ± 0.13	0.69 ± 0.05
E. flava	E trap[c]	1 (7)	14.91 ± 2.46	1.00 ± 0.07

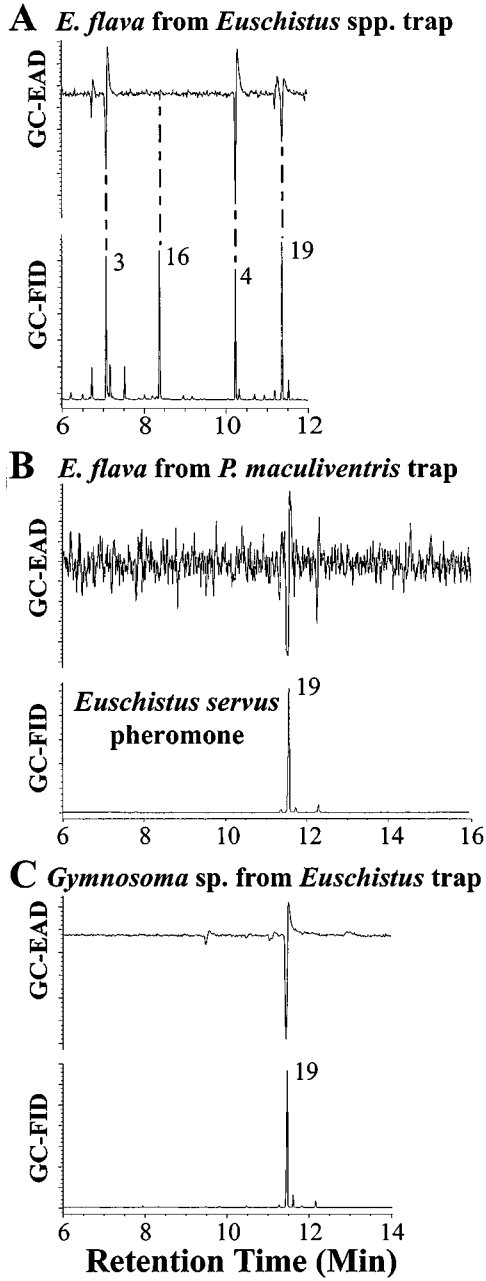
[a] Pheromone codes as defined in text; SSB = spined soldier bug.
[b] Data are presented for flies collected during the 1999 season; EAD data (not shown) for *H. aurata* field-collected in 2000, and *E. flava* reared from SSB in 2000 and 2001, are consistent with these data.
[c] Collected October 11, 2001, in trap baited with methyl (*E*,*Z*)-2,4-decadienoate.

EAD ratio of 1.00 ± 0.07 (Table 1), and its antennae were sensitive to methyl (*E*,*Z*)-2,4-decadienoate (Figure 5A). An *E. flava* female from a PM-baited trap appeared to be relatively insensitive to *Euschistus servus* pheromone (Figure 5B), while a common *Euschistus* tachinid parasitoid (*Gymnosoma* sp.) is highly sensitive to methyl (*E*,*Z*)-2,4-decadienoate (Figure 5C). Reexamination of the EAD data for *E. flava* in the 1999 season revealed that 16 of 19 flies (Table 1; PM/PMO flies) exhibited low response ratios for α-terpineol/linalool (range: 0.88–2.46; mean ± SEM: 1.55 ± 0.09), whereas 3 of these 19 flies were much less sensitive to linalool resulting in higher α-terpineol/linalool ratios (range: 3.29–9.45; mean ± SEM: 4.93 ± 0.78).

DISCUSSION

Despite revelations, largely based upon *Drosophila* research (Benzer, 1967; Pascual and Preat, 2001), of the genetic foundations for insect (and all animal) behavior, the mechanism whereby a "simple" insect parasitoid acquires, retains, and passes on kairomone messages remains mysterious. In this study we have attempted to partially elucidate this process by electrophysiologically examining the peripheral chemosensory responses of tachinid parasitoids behaviorally associated with particular pentatomid hosts.

Affect of (E)-2-Octenal. The extreme electrophysiological sensitivity of the tachinid parasitoids, *Euclytia flava* and *Hemyda aurata*, to one of the most common defensive compounds of Heteroptera, (*E*)-2-octenal (Aldrich, 1995b), inspired the field experiment to determine if inclusion of this compound with synthetic *Podisus* pheromones would alter attraction of these parasitoids or the *Podisus* bugs themselves. The results in this regard are clear for the bugs; inclusion of


A E. flava from Euschistus spp. trap
GC-EAD
GC-FID
3
16
4
19
6
8
10
12
B E. flava from P. maculiventris trap
GC-EAD
GC-FID
Euschistus servus
pheromone
19
6
8
10
12
14
16
C Gymnosoma sp. from Euschistus trap
GC-EAD
GC-FID
19
6
8
10
12
14
Retention Time (Min)

(*E*)-2-octenal tended to counteract attraction of both *P. maculiventris* and *P. neglectus* adults to pheromone. The affect of adding (*E*)-2-octenal to pheromone is less obvious for the tachinids. When (*E*)-2-octenal was added to the pheromone of *P. maculiventris*, attraction of *E. flava* was depressed or unaffected, whereas traps baited with (*E*)-2-octenal and *P. neglectus* pheromone tended to attract more *E. flava* females. It seems possible that these unusual results are somehow due to the fact that *P. neglectus* is a rare species compared to the spined soldier bug so that, although (*E*)-2-hexenal and linalool are strongly EAD-active, the synergism between these compounds at the central nervous system level is weak, resulting in the kairomonal effect of (*E*)-2-octenal being dominant. For *H. aurata*, the EAD data clearly indicate that this species is much more sensitive at the peripheral level to (*E*)-2-octenal than is *E. flava*. Unfortunately, *H. aurata* seldom entered the traps used in this study, so future tests using more effective traps need to be conducted to clarify this situation.

Tachinid Kairomone Strains. The seemingly conflicting affect of (*E*)-2-octenal on the attraction of *E. flava* could be interpreted as evidence that kairomone-strains of this parasitic fly exist based on differential sensitivity to (*E*)-2-octenal. However, this seems unlikely based on the near identical EAD sensitivities to key kairomone compounds for flies associated with either *P. neglectus* or *P. maculiventris*.

The evidence that kairomone strains of *E. flava* actually do exist in nature based on the pheromones of *Podisus* and *Euschistus* pentatomids is more convincing. The pheromones of these stink bugs are chemically dissimilar (Aldrich, 1995b), suggesting that cross-attraction is improbable. Furthermore, prior data from the same field site used for the present study verified that *Euschistus* adults are acceptable, albeit nonpreferred hosts for *E. flava* (Aldrich et al., 1991b). The observation that an *E. flava* female collected in a *Euschistus* pheromone-baited trap was almost totally EAD-insensitive to linalool, combined with the finding that an *E. flava* female collected in a *P. maculiventris* pheromone-baited trap only weakly responded to *E. servus* pheromone by GC-EAD, is direct evidence for coexisting kairomone strains of this tachinid. Reexamination of the EAD data for *E. flava* showed that a few flies caught in *P. maculiventris* pheromone-baited traps were anomalous in that they were relatively insensitive to linalool, although not so insensitive as the *Euschistus*-associated individual. Apparently ample chemosensory

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FIG. 5. Gas chromatogram–electroantennogram detector experiments for (A) antennae of a *Euclytia flava* female collected in a *Euschistus* spp. pheromone-baited trap to (*E*)-2-octenal (**3**), linalool (**16**), α-terpineol (**4**) and methyl (*E*,*Z*)-2,4-decadienoate (**19**) standards (10 ng/μl CH_2Cl_2); (B) antennae of *E. flava* female collected in a *Podisus maculiventris* pheromone-baited trap to a pheromone extract of *Euschistus servus* male pheromone; and (C) antennae of female *Gymnosoma* spp. reared from *E. tristigmus* to methyl (*E*,*Z*)-2,4-decadienoate (**19**) (10 ng/μl CH_2Cl_2) (DB-WAXetr column for all GC runs; $\sim$3 μl/injection).

variation exists in the population of *E. flava* for natural selection of kairomone-strains of parasitoids. Similarly, Dietrick and Bosch (1957) observed that, "The larvaevorid fly *Trichopoda pennipes* Fabr. [Tachinidae], a parasite of hemipterous insects, apparently exists in the United States in at least three host-determined strains." Our preliminary GC-EAD experiments with host-associated strains of *T. pennipes* (Zhang and Aldrich, unpublished data) are consistent with the results presented here for *E. flava*, together suggesting that the intensity of a tachinid's antennal response toward particular host volatiles is directly proportional to host acceptability.

Summary and Significance. Clearly the tachinid parasitoids studied here, especially the generalist *E. flava*, have thoroughly subverted both the defensive and communicative chemical systems of their hosts to become "evolutionary boomerang" signals known as kairomones (Blum, 1974). However, the results of an earlier study of *E. flava* parasitism, also made possible by "pheromone-harvesting" wild flies, showed that this semiochemical exploitation is not unidirectional because native host species secrete repellent or masking compounds that confer some degree of immunity (lacking in exotic species) to oviposition by tachinids (Aldrich, 1995a).

From a practical point of view, application of today's GC-EAD technology may help avoid future mistakes in classical biological control, such as the errant attempt to control the cosmopolitan stink bug, *Nezara viridula* L., in Hawaii with what may have been a kairomonally incompatible strain of a *Trichopoda* parasitoid (references in Pickett et al., 1996). Of longer range practical and theoretical interest is the question of how parasitoid host-shifts evolve, for if this process were known better it might be possible to devise artificial selection regimes, incorporating appropriate semiochemicals, to more quickly recreate this normally slow natural process. Such artificial selection could be used to create kairomone-strains of physiologically competent endemic beneficials that would recognize alien hosts. Theoretically, this "nonclassical" biocontrol approach should exploit the vulnerability of exotic species, which lack the coevolved antiparasitoid defenses of native host species (Hokkanen and Pimentel, 1989).

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FIELD RESPONSE OF *Ips paraconfusus*, *Dendroctonus brevicomis*, AND THEIR PREDATORS TO 2-METHYL-3-BUTEN-2-OL, A NOVEL ALCOHOL EMITTED BY PONDEROSA PINE

DENNIS W. GRAY*

Department of Ecology and Evolution
State University of New York
Stony Brook, New York 11794–5245, USA

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Abstract—Methylbutenol (MBO) is a major component of the aggregation pheromone of the European spruce beetle *Ips typographus* and also has been found to be emitted in large amounts by several species of pine native to western North America. This study investigates the influence this signal may have on the behavior of North American bark beetles and examines whether MBO functions as a defensive compound for emitting pines. The response of two North American bark beetles (*Ips paraconfusus* and *Dendroctonus brevicomis*) and their predaceous beetles (Trogositidae and Cleridae) to MBO, pheromone, and monoterpenes in varying release rates was investigated in the field using Lindgren funnel traps. MBO exhibited no repellent properties when tested alone, nor did MBO appear to have any effect on the aggregation response of these bark beetles and their predators to their pheromones. These results provide no support for a defensive function of MBO.

Key Words—Bark beetle, methylbutenol, MBO, 2-methyl-3-buten-2-ol, pheromone, *Ips paraconfusus*, *Dendroctonus brevicomis*, Cleridae, Trogositidae, Scolytidae, monoterpene, predator, plant defense, Ponderosa pine, *Pinus ponderosa*.

INTRODUCTION

Among the insect pests of conifers, the bark beetles of the family Scolytidae are by far the most important and destructive. During outbreaks, bark beetles may kill large numbers of trees within a given stand, and timber losses caused by bark

* E-mail: denwgray@life.bio.sunysb.edu

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beetles during single outbreaks can range into the billions of board feet (Miller and Keen, 1960; Furniss and Carolin, 1977). The destructive potential of bark beetles stems largely from their use of chemical cues and signals that allow them to choose weakened hosts and to coordinate mass attacks. Understanding the ecological chemistry of bark beetle behavior and the role of volatile compounds in modulating this behavior is crucial to both basic questions in plant–insect interactions and to developing protocols for minimizing the economic impacts of these insects.

The chemical mediation of bark beetle behavior has received extensive study for more than 30 years; and researchers have described several distinct phases of these attacks (Stark, 1981; Wood, 1982). The initial attack on a tree is made by a small number of pioneer beetles that alight upon the bark and begin burrowing tunnels into the bole. Host choice at the initial selection phase has been ascribed to attraction to host volatiles (Rudinsky et al., 1971; Miller and Borden, 1990), visual cues (Henson, 1962), or random landing behavior (Burnell, 1977; Moeck et al., 1981). These pioneer beetles then begin emitting aggregation pheromones that attract conspecific beetles to the source (Byers, 1988). Some species such as *D. pseudostugae* and *D. frontalis* are capable of producing pheromones immediately upon landing (Renwick and Vité, 1968; Vité and Renwick, 1968; Ryker et al., 1979), whereas others such as *Trypodendron lineatum* and *I. paraconfusus* appear to produce pheromones after tunneling and feeding on the host (Wood and Bushing, 1963; Pitman et al., 1965; Chapman, 1966; Borden and Slater, 1969). Emission of these aggregation pheromones results in a mass attack that can overwhelm the tree's resin defenses and even lead to its death. The pheromones are sometimes synthesized by the bark beetle by using host monoterpenes as precursors (Hughes, 1974, 1975; Hendry et al., 1980; Byers, 1981) or are synthesized *de novo* by the bark beetle (Ivarsson et al., 1993; Seybold et al., 1995). Following the successful mass attack, some species enter a third phase in which the bark beetles begin emitting different pheromones that repel additional beetles from the attacked host. Schlyter et al. (1989) showed that the production of ipsenol by *Ips typographus* inhibited the aggregation response of this insect. Emission of the antiaggregation pheromones has been interpreted as a mechanism for preventing overcrowding and competition for larval food resources.

Although the role of insect-derived pheromones in mediating bark beetle behavior has received extensive study (Borden, 1985), the role of host volatiles in mediating bark beetle behavior is less clear. Host monoterpenes have been shown to both attract (Rudinsky et al., 1971; Miller and Borden, 1990; Hobson et al., 1993) and inhibit the attraction (Hayes et al., 1994) of bark beetles. Green leaf volatiles have also been shown to inhibit the attraction (Wilson et al., 1996), and volatiles present in non-hosts have been shown to disrupt the response of bark beetles to attractant-baited traps (Huber and Borden, 2001).

Recently 2-methyl-3-buten-2-ol (MBO) was discovered to be a major component of the volatiles emitted by several species of pine native to western North

America (Harley et al., 1998). For example, in ponderosa pine, *Pinus ponderosa* Laws, MBO emissions exceeded the emission of monoterpenes by a factor of 2–10, and represented an energetic investment of 0.5% of the tree's net photosynthetic carbon assimilation (Lerdau and Gershenzon, 1997; Schade et al., 2000). However, unlike monoterpenes, MBO is not stored in plant tissues and is instead emitted immediately upon production (Harley et al., 1998). Such a large investment of energy and carbon into MBO production, relative to monoterpene production, by MBO-emitting pines suggests that this compound may serve a biological function to the emitting plant and that insect pests (and/or their predators) may use MBO for host (prey) detection.

In addition to its synthesis by some North American pines, MBO production also occurs in the European spruce bark beetle, *Ips typographus*, and is used by this insect as part of its aggregation pheromone (Bakke, 1977). The MBO produced by *I. typographus* is synthesized *de novo* by the insect (Lanne et al., 1989) and not sequestered from its host plant, Norway spruce (*Picea abies*), which does not manufacture MBO. In fact, no MBO emitting plants have yet been discovered in Europe where two bark beetles (*Ips typographus* and *Orthotomicus erosus*) use MBO as parts of their pheromone systems (Bakke, 1976; Giesen et al., 1984; Harley et al., 1998). A complete survey of the pines native to Europe has shown that none manufacture MBO (Harley et al., 1998; Gray unpublished data). In contrast, in North America, where several pines produce MBO, MBO has not been reported as a major component of the pheromone system of any North American bark beetle. It has, however, been reported as a minor pheromone component for *Polygraphus rufipennis* and may possess anti-attractant properties for *Dendroctonus rufipennis* (Werner and Holsten, 1995).

The large energetic investment in MBO production made by MBO producing pines, the appearance of MBO as a bark beetle pheromone, and some indications that MBO possesses anti-attractant properties, led to the hypothesis that in North America MBO may function as a defensive compound protecting emitting trees against bark beetle attack. MBO might serve as a defensive compound in two fashions: directly by influencing bark beetle behavior or indirectly by attracting predators and parasitoids to the emitting tree. Since it is not stored in plant tissues or manufactured in the wood, any direct defensive properties exhibited by MBO probably influence host location and acceptance behavior rather than larval survival.

MBO may have repellent properties similar to those found for 4-allylanisole (Hayes et al., 1994; Hobson, 1995), or it may inhibit landing behavior similar to the landing inhibition that Schlyter et al. (1987b) observed for *I. typographus* in response to high MBO concentrations. MBO might also inhibit the aggregation response of bark beetles. Such a response was shown by Birch and Wood (1975) when they observed that the pheromones of *I. pini* inhibited the aggregation response of *I. paraconfusus* and vice versa. In addition to these potential direct influences, MBO may benefit an emitting tree indirectly by attracting predators

and parasitoids. In Europe, bark beetle predators have been shown to be attracted to MBO (Bakke and Kvamme, 1981), and in North America the pheromones of several bark beetles have been shown to attract the predators of bark beetles (Kohnle and Vité, 1984; Mizell et al., 1984; Herms et al., 1991).

To help elucidate the function of MBO emissions, we examined the response of two North American scolytid bark beetles (*Ips paraconfusus* and *Dendroctonus brevicomis*) and predatory beetles in the families Cleridae and Trogositidae that feed on bark beetles to differing MBO release rates alone or in the presence of attractive monoterpenes or bark beetle pheromone. *I. paraconfusu*s is a marginally aggressive *Ips* found in North America that feeds on a broad range of pines (including *P. ponderosa*) and occasionally attacks and kills live trees. *D. brevicomis* is one of the most aggressive tree-killing bark beetles in North America (Furniss and Carolin, 1977), and feeds predominantly on *P. ponderosa*. The impact of MBO on bark beetle and predatory beetle behavior was examined by using field trapping experiments; insect capture rates were then used to determine whether MBO aids either plant defense against herbivores or insect location of suitable trees.

METHODS AND MATERIALS

Field-trapping experiments were conducted in the central Sierra Nevada mountains of California at the UC Berkeley Blodgett Forest Research station (38°53′, 42.9″N, 120°37′, 57.9″W). Lindgren funnel traps (Lindgren, 1983) were placed in small clearcuts located in compartments 260 (1999), and 400 (2000). These sites were logged the previous year and the slash was piled and burned the previous winter in compartment 260 or treated with a rotary masticator the previous fall in compartment 400 prior to conducting the trapping studies. The trapping sites were surrounded by second-growth mixed-conifer forest (approximately 80 years old) containing Douglas fir (*Pseudotsuga menziesii*), Ponderosa pine (*Pinus ponderosa*), sugar pine (*Pinus lambertiana*), incense cedar (*Calocedrus decurrens*), and white fir (*Abies concolor*) in roughly equal proportions, and scattered individuals of California black oak (*Quercus kellogii*). In both years and at both sites, trees within 1 km of the trapping sites were observed to be attacked and killed by bark beetles.

The experiments conducted were fully factorial designs with two levels of attractant bait (presence or absence) and four levels of MBO release. Each attractant bait by MBO release rate combination was replicated once within the trapping array ($N = 2$) and additional replication was achieved by sampling repeatedly through time. Sampling durations ranged from one to seven days depending on bark beetle flight intensity. Longer sampling durations were used during periods of low bark beetle activity to avoid having to analyze sampling periods with no captures in any treatment. MBO and monoterpenes were released from 2 ml microcentrifuge tubes suspended from the middle of the Lindgren funnel trap. Release rates were

TABLE 1. DESCRIPTION OF TREATMENTS, METHYLBUTENOL RELEASE RATES, AND MBO-EMITTING DEVICES USED IN EXPERIMENTS

Treatment	Nominal release rate (mg/day)[a]	Design
None	0	NA
Low	2.5	2-ml polypropylene Eppendorf tube fitted with a 5-ul capillary tube inserted through the cap
Medium	5	Two 2.5 mg/day releasers
High	50	2-ml polypropylene Eppendorf tube with a 1.59-mm hole drilled in cap
Very high	1000	2-ml polypropylene Eppendorf tube without cap

[a] Nominal release rates were determined by measuring weight lost from emitting devices held at 25°C in the laboratory.

varied by drilling holes of various sizes in the cap of the microcentrifuge tube, fitting a glass microcapillary tube in the cap, or leaving the tube uncapped. MBO releasing devices were constructed to achieve release rates corresponding to the MBO release rates shown to influence the behavior of *Ips typographus* by Schlyter et al. (1987a). Nominal release rates of these devices were determined by measuring volatilization gravimetrically from devices held at 25°C. Release rates and device descriptions are shown in Table 1. These release rates fall within the range of MBO levels that bark beetles are likely to encounter from phytogenic sources in the field. Leaf level MBO emission rates have been measured as high as 3.44 mg MBO/g needle tissue/day in Ponderosa pine (Gray, unpublished data), thus allowing a single fascicle to equal the release rate of the lowest MBO-emitting device. At the other end of the scale, canopy-level MBO emission rates have been measured between 172 and 344 mg MBO/m^2/s (Baker et al., 1999, 2001; Schade et al., 2000).

Sixteen eight-unit funnel traps were arrayed in four groups of four traps (Figure 1), with a spacing of 6 m between traps within a trap group and 12 m between traps in adjacent trap groups. Each trap within a group contained the same level of attractant bait, but contained a device releasing MBO at a different rate (Table 1). The collecting cups were filled with soapy water in which the captured insects drowned. This prevented captured predatory beetles from consuming the captured bark beetles. At the end of each sampling period, the traps within each group and the groups within the array were rotated clockwise to minimize the effect of trap position on the results. Captured insects were frozen or preserved in ethanol until identified and counted.

Three experiments tested whether MBO affected the behavior of bark beetles or their predators and whether MBO mediated the response of bark beetles or their predators to pheromone signals. In experiment 1, a multicomponent *Ips paraconfusus* lure (Phero Tech Inc., Delta, British Columbia) containing ipsenol, ipsdienol, and *cis*-verbenol was used as the attractant bait, and the MBO release

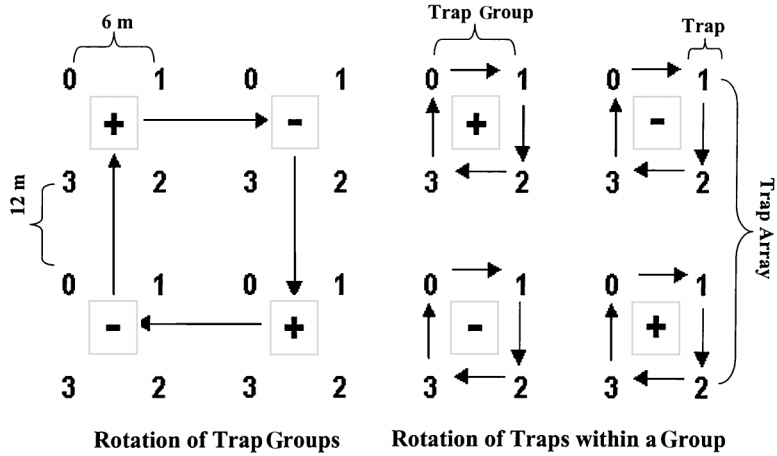


FIG. 1. Funnel trap layout and rotation scheme. Numbers 0–3 represent increasing MBO release rates as specified for each experiment. The +/− symbols denote the presence or absence of attractant bait (pheromone or monoterpenes) in traps within a trap group. Traps were spaced 6 m apart within a trap group and 12 m between traps in adjacent groups.

rates used were none, low, medium, or high (Table 1). Experiment 2 also used the *Ips paraconfusus* lure, but replaced the low MBO release rate with the very high treatment. Experiment 3 used a multicomponent *Dendroctonus brevicomis* lure (Phero Tech Inc., Delta, British Columbia) containing *exo*-brevicomin, frontalin, and myrcene as the attractant bait; and the MBO release rates were none, medium, high, and very high (Table 1).

A fourth experiment tested whether MBO affected the behavior of bark beetles or their predators to attractive host monoterpenes in the absence of a pheromone signal. In this experiment, host monoterpenes previously shown to be attractive to bark beetles (Rudinsky et al., 1971; Borden, 1985; Miller and Borden, 1990) were used instead of pheromones as the attractive bait. The MBO release rates were none, medium, high, and very high (Table 1). Monoterpenes were released from open-top 2 ml polypropylene microcentrifuge tubes at the following nominal rates: α-pinene = 0.47 g/day, β-pinene = 0.49 g/day, myrcene = 0.24 g/day.

Insects captured in the funnel-trap collection cups were collected at the end of each sampling period and frozen or preserved in ethanol until they were identified and counted. Scolytid bark beetles were identified to species, and all other captured insects were identified to family. The effects of attractant bait and MBO release rate on captures per sampling period were analyzed by three-way ANOVA using the GLM procedure of SAS (1990). The sampling period was included in the model as a covariate to take into account differences in sampling period duration (one to seven days) and differences in climatic conditions across samplings periods

such as temperature, cloud cover, and wind that may alter bark beetle activity levels (Bennett and Borden, 1971; Coster et al., 1978; Borden, 1981). Data were $\log_{10}(X + 1)$ transformed to meet the ANOVA assumptions of normality and homoscedasticity (Sokal and Rohlf, 1995).

RESULTS

In experiment 1, the addition of pheromone bait to traps increased the capture rate of *Ips paraconfusus* by a factor of more than 100. However, the addition of MBO did not alter the magnitude or pattern of captures across traps (Table 2, Figure 2). Predatory beetles in the families Cleridae and Trogositidae were not influenced by either *Ips paraconfusus* pheromone or MBO release rate (Table 2).

Similar results were observed in experiment 2 despite the addition of a much larger MBO release rate treatment. Pheromone bait increased bark beetle captures by a factor of more than 100; however, no effect of MBO release rate on captures of *Ips paraconfusus* could be detected (Table 3, Figure 3). No influence of either

TABLE 2. ANOVA ON EFFECTS OF SAMPLING DATE, PHEROMONE PRESENCE/ABSENCE, AND MBO RELEASE RATE ON CAPTURES OF *Ips paraconfusus*, CLERIDAE, AND TROGOSITIDAE AT LINDGREN FUNNEL TRAPS IN EXPERIMENT 1

Taxa	Source	*df*	MS	*F*	*P*
Ips paraconfusus					
	Main effects				
	Day	2	0.03836	0.4800	<0.6208
	Pheromone	1	256.856	3232.73	<0.0001
	MBO[a]	3	0.07976	1.0000	<0.4017
	Interactions				
	Pheromone × MBO	3	0.08548	1.0800	<0.3709
Cleridae					
	Main effects				
	Day	2	0.0100094	1.00	<0.3774
	Pheromone	1	0.0100094	1.00	<0.3236
	MBO[a]	3	0.0100094	1.00	<0.4034
	Interactions				
	Pheromone × MBO	3	0.0100094	1.00	<0.4034
Trogositidae					
	Main effects				
	Day	2	0.070066	1.14	<0.3315
	Pheromone	1	0.090085	1.46	<0.2342
	MBO[a]	3	0.063393	1.03	<0.3909
	Interactions				
	Pheromone × MBO	3	0.036701	0.60	<0.0622

[a] MBO release rates as in Table 1 with "very high" treatment omitted.

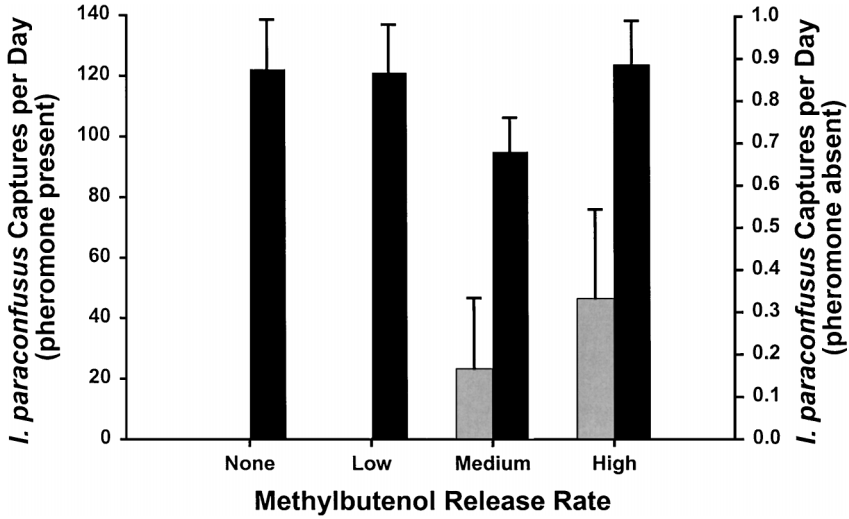


FIG. 2. Catches of *Ips paraconfusus* in eight-unit Lindgren funnel traps baited with varying release rates of MBO and with (black bars) or without (gray bars) *I. paraconfusus* pheromone in experiment 1. Error bars represent ±1 standard error.

pheromone or MBO release could be detected on the capture rates of predatory clerids or trogositids (Table 3).

In experiment 3, the addition of *D. brevicomis* pheromone caused a significant increase in *D. brevicomis* and trogositid captures. On the other hand, MBO release rate did not change *D. brevicomis* or trogositid capture rates in either the presence or absence of pheromone (Table 4, Figure 4). Neither pheromone nor MBO release rate changed the capture rates of predacious clerid beetles (Table 4).

In experiment 4, where host monoterpenes were used as the attractant bait, the number of bark beetles captured during the trapping period was quite low, and no differences were seen among treatments. Analysis of the predatory beetles captured showed that neither monoterpenes nor MBO release rate affected clerid or trogotidid captures (Table 5, Figure 5).

DISCUSSION

The results of the trapping experiments did not support the hypothesis that MBO emissions from Ponderosa pine provide protection against bark beetle attack. The data showed no indication that *I. paraconfusus* or *D. brevicomis* were repelled by MBO alone, or by MBO in combination with host monoterpenes or bark beetle pheromone. Similarly, the major insect predators of these bark beetles were not attracted by MBO, suggesting that MBO does not provide an indirect defense.

TABLE 3. ANOVA ON EFFECTS OF SAMPLING DATE, PHEROMONE PRESENCE/ABSENCE, AND MBO RELEASE RATE ON CAPTURES OF *Ips paraconfusus*, CLERIDAE, AND TROGOSITIDAE AT LINDGREN FUNNEL TRAPS IN EXPERMENT 2

Taxa	Source	*df*	MS	*F*	*P*
Ips paraconfusus					
	Main effects				
	Day	3	5.6594	17.54	<0.0001
	Pheromone	1	166.47	515.83	<0.0001
	MBO[a]	3	0.2065	0.64	<0.5927
	Interactions				
	Pheromone × MBO	3	0.2271	0.70	<0.5539
Cleridae					
	Main effects				
	Day	3	0.00751	1.00	<0.4001
	Pheromone	1	0.00751	1.00	<0.3219
	MBO[a]	3	0.00751	1.00	<0.4001
	Interactions				
	Pheromone × MBO	3	0.00751	1.00	<0.4001
Trogositidae					
	Main effects				
	Day	3	0.0100	0.65	<0.5838
	Pheromone	1	0.0000	0.00	<1.0000
	MBO[a]	3	0.0100	0.65	<0.5838
	Interactions				
	Pheromone × MBO	3	0.0200	1.31	<0.2813

[a] MBO release rates as in Table 1 with "low" treatment omitted.

Despite the lack of evidence that MBO possesses defensive properties, it should be noted that this study examined only two of the many species of bark beetle found in North American pine forests. While *I. paraconfusus* is one of the most aggressive *Ips* in North America and attacks a wide range of pines, it is much less aggressive than its European congeners (especially *I. typographus*). Nonetheless, *I. paraconfusus* is an aggressive killer of pole-size trees and the tops of mature trees, especially *P. ponderosa* in the central Sierra Nevada mountains where this study was conducted (Furniss and Carolin, 1977). *D. brevicomis*, on the other hand, is one of the most destructive bark beetles present in North America and frequently attacks healthy trees. However, since both species of pine (*P. ponderosa* and *P. coulteri*) attacked by *D. brevicomis* produce MBO (Harley et al., 1998), its failure to respond to MBO may indicate an adaptation to feeding on these hosts. If, in fact, MBO has a defensive function, it likely has its effect on those bark beetle species that do not feed on MBO-producing trees, and this may help account for host specificity among bark beetle species. Perhaps MBO production excludes *P. ponderosa* from the host range of Scolytids that feed on non-MBO-producing

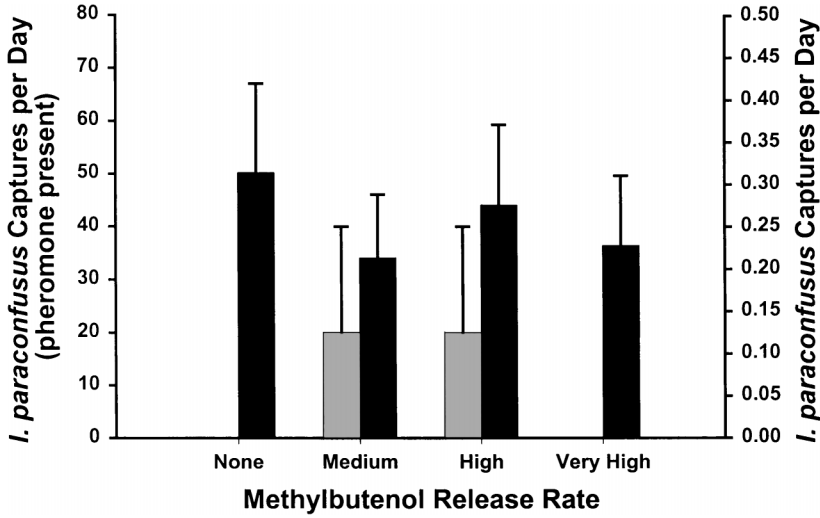


FIG. 3. Catches of *Ips paraconfusus* in eight-unit Lindgren funnel traps baited with varying release rates of MBO and with (black bars) or without (gray bars) *I. paraconfusus* pheromone in experiment 2. Error bars represent ±1 standard error.

TABLE 4. ANOVA ON EFFECTS OF SAMPLING DATE, PHEROMONE PRESENCE/ABSENCE, AND MBO RELEASE RATE ON CAPTURES OF *Dendroctonus brevicomis*, CLERIDAE, AND TROGOSITIDAE AT LINDGREN FUNNEL TRAPS IN EXPERMENT 3

Taxa	Source	*df*	MS	*F*	*P*
Dendroctonus brevicomis					
	Main effects				
	Day	9	2.39077	7.91	<0.0001
	Pheromone	1	116.73	386.39	<0.0001
	MBO[a]	3	0.15709	0.52	<0.6692
	Interactions				
	Pheromone × MBO	3	0.04864	0.16	<0.9224
Cleridae					
	Main effects				
	Day	9	0.04671	1.00	<0.4442
	Pheromone	1	0.04804	1.03	<0.3126
	MBO[a]	3	0.05205	1.11	<0.3462
	Interactions				
	Pheromone × MBO	3	0.02402	0.51	<0.6736
Trogositidae					
	Main effects				
	Day	9	1.10599	5.61	<0.0001
	Pheromone	1	11.8484	60.07	<0.0001
	MBO[a]	3	0.25450	1.29	<0.2801
	Interactions				
	Pheromone × MBO	3	0.33673	1.71	<0.1682

[a] MBO release rates as in Table 1 with "low" treatment omitted.

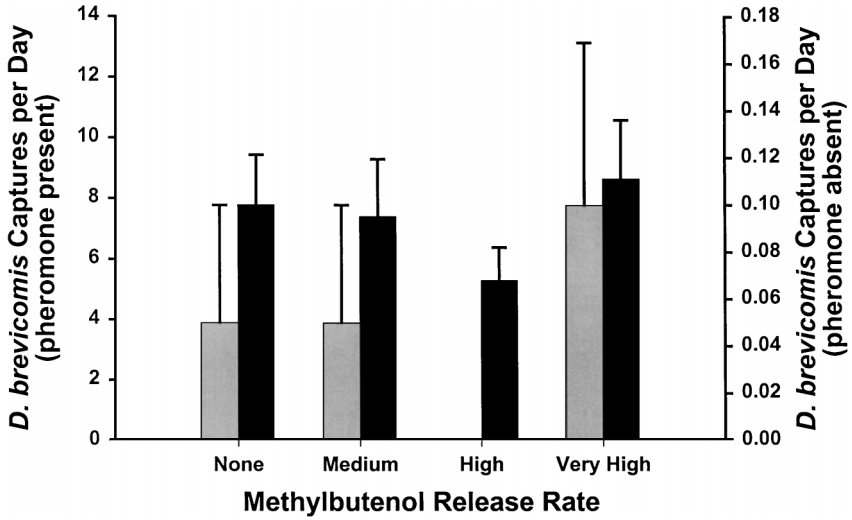


FIG. 4. Catches of *Dendroctonus brevicomis* in eight-unit Lindgren funnel traps baited with varying release rates of MBO and with (black bars) or without (gray bars) *D. brevicomis* pheromone in experiment 3. Error bars represent ±1 standard error.

TABLE 5. ANOVA ON EFFECTS OF SAMPLING DATE, MONOTERPENE PRESENCE/ABSENCE, AND MBO RELEASE RATE ON CAPTURES OF PREDACEOUS CLERIDAE AND TROGOSITIDAE AT LINDGREN FUNNEL TRAPS IN EXPERMENT 4

Taxa	Source	*df*	MS	*F*	*P*
Cleridae					
	Main effects				
	Day	1	0.6851	2.88	<0.1032
	Monoterpene	1	0.0086	0.04	<0.8512
	MBO[a]	3	0.1269	0.53	<0.6647
	Interactions				
	Monoterpene × MBO	3	0.1481	0.62	<0.6075
Trogositidae					
	Main effects				
	Day	1	0.1059	0.26	<0.6168
	Monoterpene	1	0.3693	0.90	<0.3532
	MBO[a]	3	0.1264	0.31	<0.8199
	Interactions				
	Monoterpene × MBO	3	0.1533	0.37	<0.7735

[a] MBO release rates as in Table 1 with "low" treatment omitted.

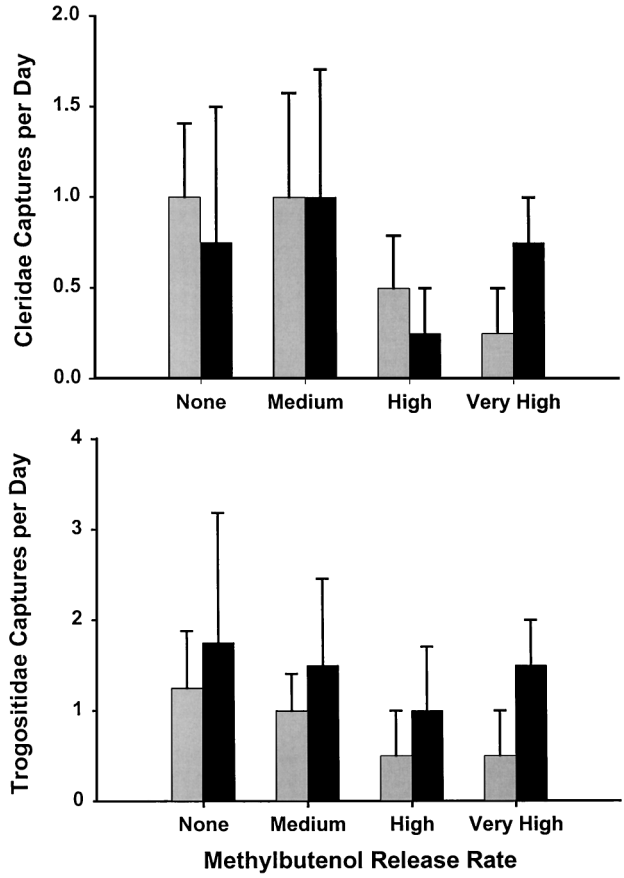


FIG. 5. Catches of predacious beetles in the families Cleridae and Trogositidae in eight-unit Lindgren funnel traps baited with varying release rates of MBO and with (black bars) or without (gray bars) monoterpenes in experiment 4. Monoterpenes were released at nominal rates of: α-pinene = 0.47 g/day, β-pinene = 0.49 g/day, myrcene = 0.24 g/day. Error bars represent ± 1 standard error.

conifers (*A. concolor*, *P. menziezii*, *C. decurrens*, and *P. lambertiana*). However, scolytid species that feed exclusively on non-MBO-producing conifers have not been screened for their response to MBO.

A spatial discontinuity also exists between the site of MBO production and bark beetle attack. MBO production only occurs in the photosynthetic tissues, whereas bark beetles attack the non-photosynthetic trunks. For this reason, it is possible that bark beetles never encounter strong MBO plumes emanating from potential host trees. However, insects feeding on the foliage or reproductive structures

of MBO-emitting pines would certainly encounter MBO. No such insect has yet been tested for its response to MBO.

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TOXIC AND AVERSIVE DITERPENES OF *Euphorbia esula*

FATHI T. HALAWEISH,[1,*] SCOTT KRONBERG,[2] MINDY B. HUBERT,[3] and JAMES A. RICE[1]

[1]*Department of Chemistry & Biochemistry*
South Dakota State University
Brookings, South Dakota 57007, USA

[2]*USDA-ARS, Northern Great Plains Research Laboratory*
P.O. Box 459, Mandan, North Dakota 58554, USA

[3]*Department of Animal & Range Sciences*
South Dakota State University
Brookings, South Dakota 57007, USA

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Abstract—Leafy spurge (*Euphorbia esula* L.), a plant introduced into the Great Plains of North America from Europe, has become a serious economic and ecological threat to the productivity of agricultural and natural areas. Cattle, the predominant livestock species on the Great Plains, as well as common wild ruminant species in this region appear to consume little if any leafy spurge. This is likely because they experience a toxic response after consuming small amounts of this plant, and they consequently learn to avoid it. Domestic sheep and goats can consume considerable amounts of leafy spurge and are used to help control it, but even these species may suffer a toxic response at high levels of intake. Toxic diterpene ingenols have been isolated from leafy spurge tissues, but compounds in this plant have not been evaluated with respect to their capacity to induce food aversion learning in ruminants. We conducted bioassay-guided fractionations of leafy spurge in an attempt to isolate toxic and aversive compounds. These bioassay-guided fractionations led to identification of ingenol and one of its diesters as two toxic compounds in leafy spurge that are potentially aversive to cattle.

Key Words—*Euphorbia esula*, leafy spurge, ingenol, diterpene, toxic, aversive, cattle.

* To whom correspondence should be addressed. E-mail: fathi_halaweish@sdstate.edu

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INTRODUCTION

Leafy spurge (*Euphorbia esula* L.) is a serious invasive weed on lands with both native and introduced vegetation on the northern Great Plains of North America. Although domestic sheep and goats usually graze this perennial forb, it appears to be aversive and toxic to cattle, and is a serious threat to cattle production (Johnston and Peake, 1960; Hein and Miller, 1992; Heemstra et al., 1999). Domestic sheep may also experience toxicity from leafy spurge ingestion at high levels of intake, after ingestion of particular plants or plants growing at certain locations (Johnston and Peake, 1960; Kronberg and Walker, 1999). Little is known about which species of wildlife eat or avoid leafy spurge, but it is generally considered to have a negative impact on wildlife habitat (Belcher and Wilson, 1989). With cattle, the predominant species of grazing livestock in North America, their avoidance of leafy spurge may be one important reason why this introduced plant from Eurasia has spread across two million acres of range and pasture lands in the continental United States. It is considered the worst noxious weed on these lands, with an economic impact in the millions of dollars annually (Leitch et al., 1996). Leafy spurge has high protein and low fiber levels during its early growth stages (Fox et al., 1991). However, it is not utilized as feed by cattle because it contains aversion-inducing chemicals that cause negative feedback after they ingest small amounts of it. Thus, they learn to avoid it (Kronberg et al., 1993). Extracts of leafy spurge were shown to cause skin irritation and weak tumor promotion, but not solitary carcinogenic activity (Seip and Hecker, 1982). Ingenol (Seip and Hecker, 1982) and several diterpene derivatives such as 3-*O*-(2*E*,4*Z*)-decadienoylingenol (*Euphorbia* factor E1), 3-*O*-(3,4,6)-decatrienoylingenol, and 3-*O*-(3,4,6,8)-dodecatetraenoylingenol were identified and shown to mediate irritant activity (Hecker, 1978). Ingenol and ingenol esters appear to induce toxicity because of their ability to mimic the function of diacylglycerol and activate protein kinase C (Hasler et al., 1992; Winkler et al., 1993). Protein kinase C is a phosphorylating enzyme that mediates cellular signal transduction for a large group of hormones and cellular effectors (Winkler et al., 1993). Other diterpenes, such as jatrophane and lathyrane derivatives, were also found in leafy spurge (Manners and Davis, 1987; Onwukaeme and Rowan, 1992).

Leafy spurge has been studied chemically and ecologically, but has had little evaluation of its aversion-inducing capacity for cattle or other ruminants. Ingenols are suspected as aversion-inducing compounds in leafy spurge, and ingenol 3-monobenzoate was found to be aversive to rats (Kronberg et al., 1995). Our study focused on the identification of aversive toxins in leafy spurge to further understand their impact on cattle. Bioassay-guided fractionations led to the identification of two compounds—ingenol and its ester—that are likely responsible for some of the aversion that cattle develop to leafy spurge.

METHODS AND MATERIALS

Plant Material. Air-dried spurge was used throughout the study. Aerial parts of *Euphorbia esula* in the seed-ripening growth stage were collected in June 1995 near Veblen in northeastern South Dakota. Plants were air-dried and ground through a 2-mm screen. Ground plant material (3.6 kg) was extracted with cold 90% methanol (6 × 6 leters). The aqueous methanol extract was concentrated under reduced pressure at 40°C and partitioned with petroleum ether (40–60°C, 6 × 1 liter), chloroform (6 × 1 liter) and acetone (6 × 1 liter). A thick viscous layer, formed at the interface of the chloroform and aqueous phases, was collected separately and designated as the middle layer.

Evaluation of Middle Layer and Petroleum Ether Extracts for Aversive Response from Cattle

Three groups of yearling cattle (5/group) were trained for a one-week period to eat for 30 min in the morning from feed boxes within individual pens. They were held off feed, but not water, overnight during this period and allowed ad libitum intake of grass hay as a group only after the morning training period. A test period immediately followed the training period. At the same time as the training period, the cattle were given oat silage as a novel feed in feed boxes within individual pens for 30 min. After consuming about 700 g of the novel feed, one group was treated with the petroleum ether extract and the other with the middle layer residue at a dosage of 0.059 g/kg body wt and 0.176 g/kg body wt, respectively (materials orally dosed in gelatin capsules), and the control group was given empty capsules. On the following day at the same time, the cattle were again offered the same novel feed for 30 min. The amount of feed consumed by each animal was recorded. Novel feed consumption on both days of the test period was compared using a repeated-measures design and PROC GLM (SAS, 1991) to determine if the cattle had an aversive response to the novel feed on the second day. Treatment group was the between-animal effect, and postgavage response was the within-animal effect. When appropriate, individual means were compared with the protected LSD procedure (SAS, 1988).

Screening of Aversive/Toxic Effect of Petroleum Ether Fractions on Rats and Brine Shrimp

The middle layer did not induce aversions in cattle, but the petroleum ether extract induced strong aversions, so this extract was investigated as follows. The petroleum ether extract (20 g) was subjected to fractionation on silica gel (G60, 300 g) using flash column chromatography (5 × 50 cm) and developed with 100%

TABLE 1. TOXIC AND AVERSIVE EFFECTS OF FRACTIONS FROM LEAFY SPURGE (*Euphorbia esula* L.)

Fractions	Solvent	%	Aversive effect[a,b]	Toxic effect[b,c]
1–4	Pet.ether–ethyl acetate	8:2	–	–
5, 6	Pet.ether–ethyl acetate	75:25	–	–
7, 8	Pet.ether–ethyl acetate	65:35	–	++++
9, 10	Pet.ether–ethyl acetate	50:50	+	++++
11	Pet.ether–ethyl acetate	25:75	+	++++
12	Pet.ether–ethyl acetate	25:75	++++	++++
13	Ethyl acetate	100	++++	++++
14, 15	Methanol	100	–	–

[a] Aversive effect measured as reduction of novel fluid intake by rats.
[b] + = 25% aversive/cytotoxic activity; ++ ++ = 100% aversive/cytotoxic activity.
[c] Toxic effect measured as percentage of brine shrimp killed.

petroleum ether with a gradient increase in polarity with ethyl acetate (Table 1). One-liter fractions were collected, concentrated with a rotary evaporator, and evaporated to dryness under nitrogen. Fifteen fractions were collected. Aversive activity of fractions 1–15 was tested with rats (dosage 30 mg/rat) to measure their response to each fraction and compare them among fractions (Kronberg et al., 1995). Data on their aversive activity for rats was coupled with data from the brine shrimp toxicity assays to determine if these were consistent with cytotoxic effects of compounds in leafy spurge. For the brine shrimp toxicity assays, 0.25 mg of each extract was added to 8-ml vials ($N = 4$) and dissolved in 20 μl of DMSO (dimethyl sulfoxide) and mixed. The brine shrimp assays were conducted as outlined by Anderson et al. (1991).

Isolation and Identification of Aversive/Toxic Compounds

Combined fractions 12 and 13 (1.21 g) were subjected to a second step of purification using flash column chromatography (5 liters, 1:1, petroleum ether–ethyl acetate) and afforded 10 fractions (500 ml each) of which fractions 8 (32.5 mg) and 9 (25.6 mg) were finally purified with normal-phase HPLC using Econosil, 5μ (4.5 × 250 mm) and Econosil 10μ (22 × 250 mm) columns. HPLC columns were developed with a gradient system using hexane and a gradual increase in percentage of isopropanol. Compounds 8.5 (3.0 mg) and 9.4 (2.0 mg) were isolated from fractions 8 and 9, respectively. Structural elucidation was achieved using spectroscopic techniques. Nuclear magnetic resonance experiments were conducted with a Bruker DRX 400 spectrometer. Mass spectra were recorded using EI and CI techniques with a Finnigan 995633. Infrared spectra were recorded on a Bio-Rad FTS-60A infrared spectrophotometer.

Cytotoxicity Assays

Evaluation of the isolated compounds for cytotoxicity was made with bovine lymphosarcoma cells (CRL-8037 BL-3, American Type Culture Collection). The culture medium was Eagle's MEM with 0.1 mM nonessential amino acids and Earle's BSS (90%) and fetal bovine serum (10%). Two milligrams of each of the isolated compounds from leafy spurge was dissolved in 400 μl dimethyl sulfoxide (DMSO). Either zero, 1, 5, 10, 25, and 50 μl of DMSO alone (control) or 0, 1, 5, 10, 25, and 50 μl of DMSO plus one of the isolated compounds were incubated with 2 ml of the cells for 18 hr at 37°C. Aliquots of cells were withdrawn after 18 hr and stained using a two-step acridine orange (AO) technique: 200 μl of cell suspension (1–2 $\times$ 10^6/ml) treated for 30 sec with 200 μl of a buffer containing 0.1% Triton and 0.08 N HCI followed by staining with AO staining buffer (6 μg AO/ml). The metachromatic AO-stained cells were measured for green flourescence (510–530 nm band-pass filter) for amounts of DNA and red fluorescence (630 nm long-pass filter) for amounts of RNA.

Evaluation of Ingenol and Ingenol 3-Benzoate for Aversive Response from Cattle

Seip and Hecker (1982) found 99 mg of ingenols in 20 kg of air-dried leafy spurge. Therefore, we assumed that 0.0005% of air-dried leafy spurge was ingenol. Previous work (Kronberg et al., unpublished data) indicated that 0.3% of body weight is an aversive dosage of air-dried leafy spurge for cattle, so treated calves were dosed with 0.0148 mg of ingenol or ingenol 3-benzoate/kg body wt. Ingenol and ingenol 3-benzoate were purchased from LC Laboratories (Woburn, Massachusetts, USA). Ingenol, the parent compound, has weak affinity for protein kinase C while ingenol 3-benzoate has considerable affinity for protein kinase C and, therefore, likely has greater toxicity to cattle. Ingenol 3-benzoate is similar to ingenol esters found in leafy spurge. The gavage solution was made by initially solubilizing 25 mg of each ingenol with 5 ml of ethanol. Fifty milliliters of deionized water was then added to each solution, and these mixtures were gavaged. Control animals received an equivalent amount of ethanol and water.

Calves were trained and managed as described for the initial cattle trial. They were given 907 g of millet as a novel feed for 15 min in the first test period for ingenol, and 907 g of sugar beet pulp pellets for 15 min in the second test period for ingenol 3-benzoate. After consuming some of the novel feed, calves were gavaged with either ingenol or ingenol 3-benzoate in the ethanol–water solution. On the morning of the following day, they were offered novel feed again to determine if they formed an aversion to it. One calf (I4) was gavaged with ingenol a second time shortly after it ate a significant amount of novel feed, indicating that ingenol had not elicited an aversion. Because of the small amounts of ingenol and ingenol 3-benzoate available, the number of calves was limited, as was the number of days

that they were treated with the compounds. Novel feed consumption on both days of the test period was compared using a repeated-measures design and PROC GLM (SAS, 1991) to determine if the cattle had an aversive response to the novel feed on the second day. Treatment group was the between-animal effect, and postgavage response was the within-animal effect. When appropriate, individual means were compared with the protected LSD procedure (SAS, 1988).

Specroscopic Data

Compound 8.5. EI-MS *m*/*z* 348, 330 (M^+-18), 313, 295 (313-18), 246, 236, 214 (100%) and 197. IR ($CHCl_3$) 3450, 2930, 1685. UV abs (MeOH) 214 nm. ^1H NMR (MeOH): δ 6.10 (1H, s, H-1), δ 3.65 (1H, s, H-3), δ 3.85 (1H, br s, H-5), δ 6.10 (1H, m, H–7), δ 4.10 (1H, m, H–8), δ 2.35 (1H, s, H–11), δ 2.25 (1H, m, H–12a), δ 1.65 (1H, m, 12b), δ 0.93 (1H, m, H–13), δ 0.92 (1H, m, H–14), δ 1.30 (1H, s, H–16), δ 1.35 (1H, s, H–17), δ 1.7 (1H, m, H–19), δ 4.10 (1H, d, H–20a, $J = 12.5$) and δ 4.25 (1H, d, H–20b, $J = 12.5$). ^{13}C NMR (MeOH) δ 12.68 (C-1), δ 139.42 (C-2), δ 79.44 (C-3), δ 84.47 (C-4), δ 73.25 (C-5), δ 143.25 (C-6), δ 127.56 (C-7), δ 42.97 (C-8), δ 206.59 (C-9), δ 42.67 (C-10), δ 23.14 (C-11), δ 30.49 (C-12), δ 22.62 (C-13), δ 22.25 (C-14), δ 37.97 (C-15), δ 15.29 (C-16), δ 15.40 (C-17), δ 17.05 (C-18), δ 28.25 (C-19), δ 62.98 (20).

Compound 9.4. EI-MS *m*/*z* 552, 538, 496, 382, 368, (100%), 348, 330, (348-18), 313, 295 (313-18), 246, 236, 214, 183, and 43 and 197. IR ($CHCl_3$) 3450, 2930, 1720, 1685. UV abs (MeOH) 214 nm.

RESULTS AND DISCUSSION

Initial screening for aversive activity showed the petroleum ether and chloroform extracts and the middle layer to be aversive to rats. Further screening of the middle layer and petroleum ether extracts on cattle showed no aversive response from cattle to the middle layer, but strong aversion to the petroleum ether extract ($P < 0.01$; Figure 1). Chromatographic separation guided by rat aversion trials and brine shrimp assays showed fractions 12 and 13 (Table 1) to have the greatest aversive and toxic effects. Further separation of the combined fractions 12 and 13 by column chromatography provided 10 fractions. Fractions 8 and 9 of the second separation constituted more than 80% of the combined original fractions 12 and 13. Preparative HPLC using normal-phase gradient analysis was conducted and monitored at a wavelength of 220 nm. All fractions were collected. HPLC purifications afforded compounds 8.5 and 9.4 as the major compounds in fractions 8 and 9.

Detailed 1D NMR (H^+, ^{13}C, and DEPT) and 2D NMR (GCOSY, HMBC, HMQC, and NOESY) experiments were conducted for compounds 8.5 and 9.4.

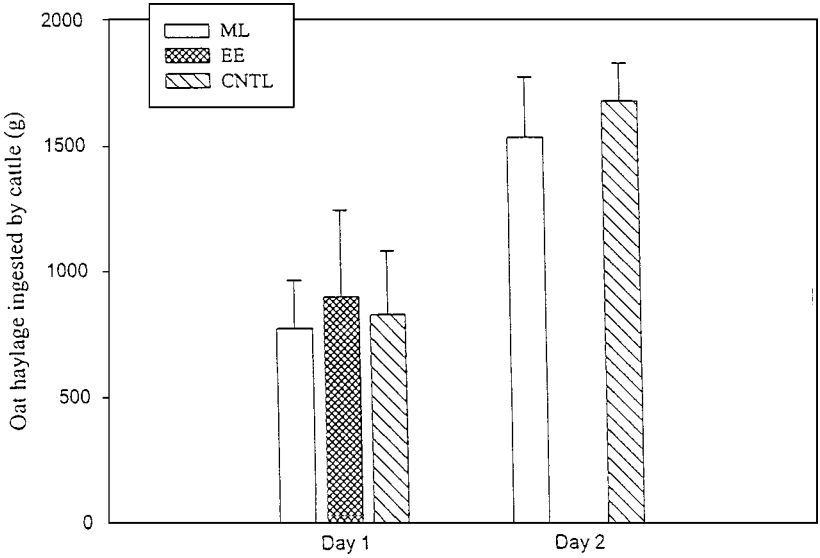


FIG. 1. Ingestion by cattle of a novel-tasting feed before and after receiving different portions of the original methanol extract of leafy spurge in gelatin capsules. The portions were a thick viscous layer at the interface between the chloroform and aqueous layers named the middle layer (ML) and the petroleum ether extract (pEE). A third group of control cattle (CNTL) received an empty gelatin capsule.

Mass spectral analysis of the two compounds showed evidence of a diterpenoid structure with molecular ion peaks at m/z 331 and 348. Mass spectral analysis showed a molecular ion peak for compound 8.5 at m/z 348 and fragments of m/z 313 and 295, which are characteristic of ingenol (Seip and Hecker, 1982). Compound 9.4 showed a molecular ion peak at m/z 330 and fragments of 313, 295, 183 and 43, which are characteristic of the ingenane skeleton and fragments of dodecanoate and acetate (m/z 183 and 43, respectively). The latter fatty acids were shown clearly from the m/z fragments of 43 and 183 corresponding to acetyl and dodecayl radicals, respectively. Figure 2 illustrates the structures of compounds 8.5 and 9.4.

H^+ and ^{13}C NMR data corresponded well with published data for the diterpene ingenol (Seip and Hecker, 1982). ^1H-^1H connectivities were confirmed from COSY and NOESY experiments. DEPT experiments assigned carbon shifts for all protonated carbons. A heteronuclear multiple bond correlation (HMBC) assigned shifts at δ 139.42 and 143.25 to quaternary carbons C-2 and C-6, respectively. NMR, MS, and IR data are in full agreement with published literature (Seip and Hecker, 1982). Therefore, compound 8.5 is identified as ingenol. NMR data for compound 9.4 could not be completed with this experiment,

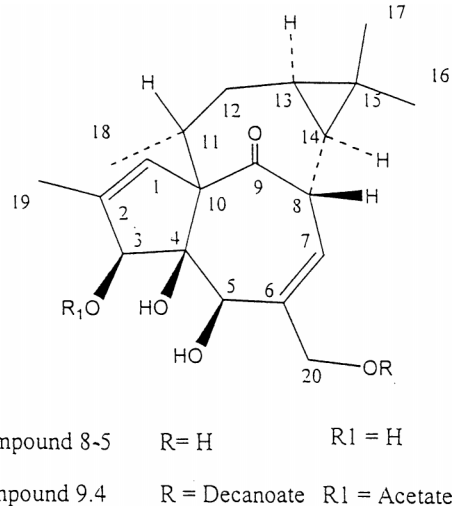


FIG. 2. Aversive diterpene ingenol (compound 8.5) and diester derivative (compound 9.4).

but the identity of it was established from mass spectral analysis. Moreover, alkaline hydrolysis of compound 9.4 followed by comparison of the diterpene part showed a complete match with ingenol in TLC and HPLC analysis. Therefore, compound 9.4 was tentatively identified as ingenol-20-dodecanoate, 3-acetate by comparison with published data (Seip and Hecker, 1982). Further NMR analyses are in progress to establish the positions of the diester derivatives of ingenol.

Compounds 8.5 and 9.4 showed toxicity to lymphosarcoma cells at 10 μl or more of DMSO plus a compound (Figures 3 and 4). The cell assay showed little difference in the cytotoxicity of compound 8.5 (ingenol, the parent diterpene alcohol) and compound 9.4 (ingenol diester derivative). Ingenol and its esters vary considerably in their biological activity (Hirata, 1975; Hirota et al., 1980). Ingenol has lower activity than some of its esters, but will still bind to protein kinase C and activate the enzyme (Hasler et al., 1992). Ingenol has been shown to be active in different cell systems with effects similar to those of the phorbol esters in respect to morphological change, cell to cell communication, epidermal growth factor binding, arachidonic acid metabolite release and ornithine decarboxylase activity (Hasler et al., 1992).

For the first trial with ingenol, change in intake of novel feed by treated and control cattle from the first to second days was similar ($P = 0.41$), and the ingenol treatment elicited an aversion in only one animal (Figure 5). This calf decreased intake of the novel feed by 83% from the first to second days. One calf that did not show an aversion from the initial ingenol treatment was dosed again and avoided

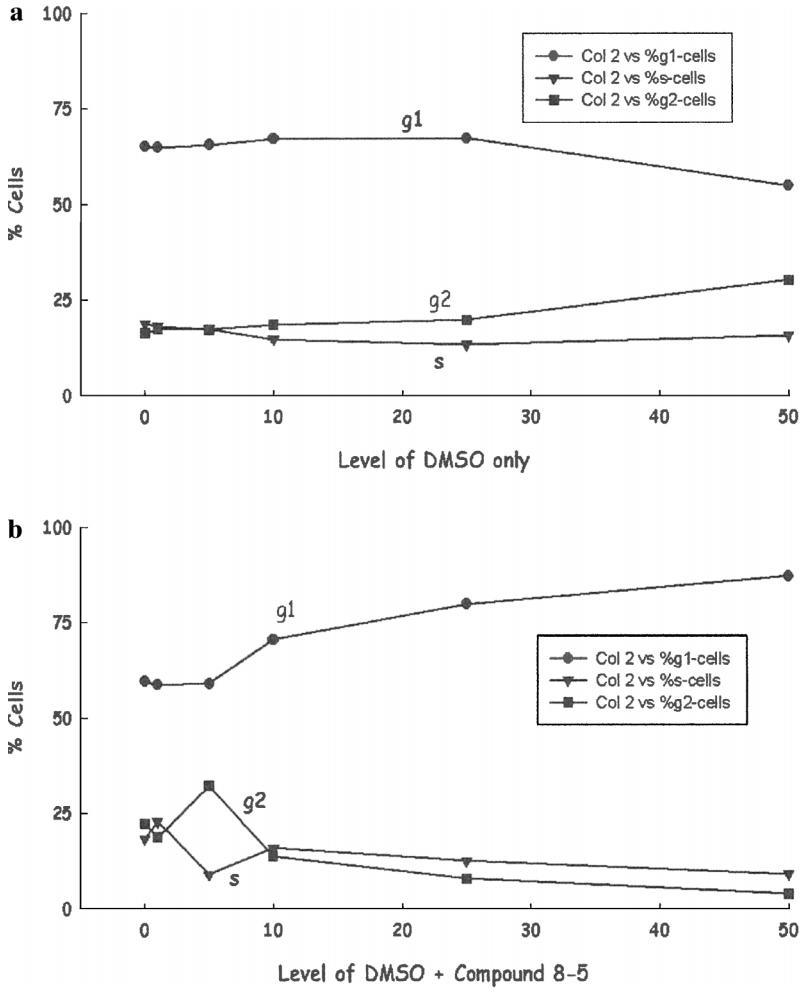


FIG. 3. Cell cycle analysis based on percent of bovine lymphosarcoma cells in each of three phases of the cell cycle: lag phase (g1), exponential phase (g2), and stationary phase (s). Effect on cell cycle when different amounts of DMSO was added to the lag phase of growing cells (a) and when different amounts of compound 8.5 dissolved in DMSO was added to the lag phase of growing cells (b).

the novel feed after a second treatment with ingenol. These results are consistent with work indicating that ingenol, the parent alcohol, is less toxic than some of its esters (Hasler et al., 1992). However, we recently determined that the air-dried leafy spurge from our South Dakota site contained at least 0.0074% ingenol. This

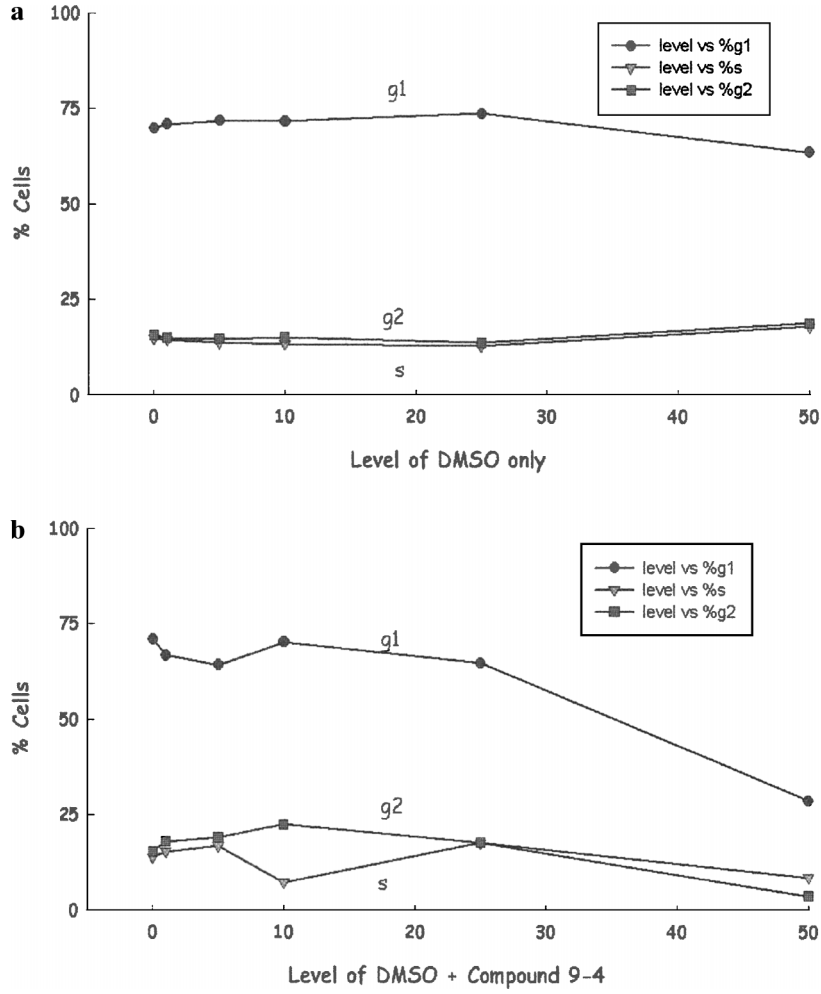


FIG. 4. Cell cycle analysis based on percent of bovine lymphosarcoma cells in each of three phases of the cell cycle: lag phase (g1), exponential phase (g2), and stationary phase (s). Effect on cell cycle when different amounts of the DMSO was added to the lag phase of growing cells (a) and when different amounts of compound 9.4 dissolved in DMSO was added to the log phase of growing cells (b).

level is considerably higher than our assumption of 0.0005% of air-dried leafy spurge that we used when determining our dosage rate for both aversion trials. Ingenol may have elicited more aversion had we used a higher dosage based on this level of ingenol in leafy spurge.

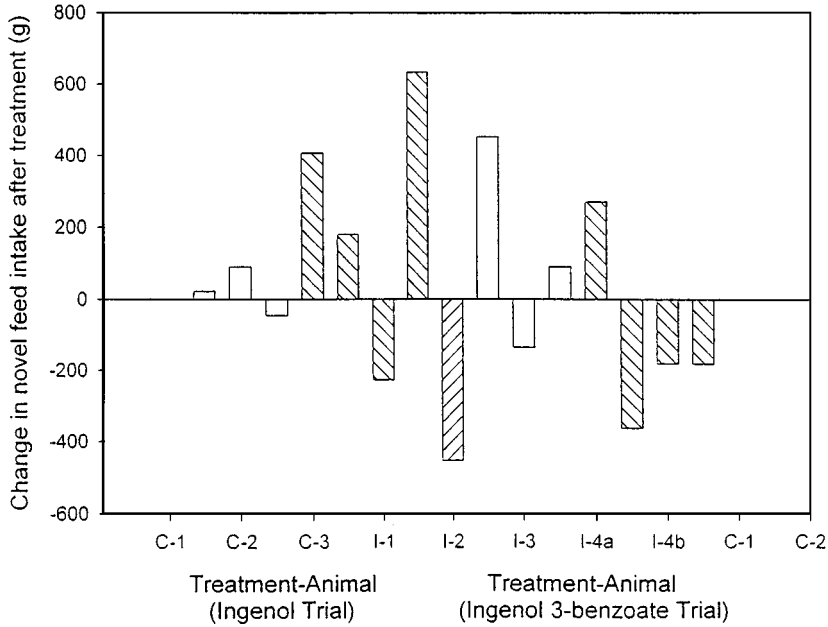


FIG. 5. Change in intake of a novel feed by cattle after gavage with ingenol (or ingenol 3-benzoate) in ethanol and water or only ethanol and water (control). Animals with "I" before their number were gavaged with ingenol or ingenol 3-benzoate (separate trials for each ingenol). Animals with "C" before their number were gavaged with only ethanol and water. Animal 940 did not show an aversion to the novel feed after the first time it was gavaged with ingenol (intake indicated at I-4a), so it was dosed again and its intake following the second gavage of ingenol is indicated by I-4b.

The esters are more lipophilic than the parent ingenol and, therefore, more likely to be absorbed through the intestinal mucosa. Ingenol 3-benzoate is similar to other ingenol esters that are in leafy spurge or possibly created by microbial metabolism of ingenols in the rumen of cattle. Hubert et al. (unpublished data) observed that extracts of 6-hr-long fermentations of leafy spurge and cattle ruminal digesta are more toxic to brine shrimp than are extracts of mixtures of leafy spurge and cattle ruminal digesta are more toxic to brine shrimp than are extracts of mixtures of leafy spurge and cattle ruminal digesta that were not allowed to ferment. One dose of ingenol 3-benzoate elicited an aversion in three of four calves tested (novel feed intakes decreased 89, 33, and 80% from the first to second day for these three calves). However, there was no significant difference ($P = 0.30$) between the control and treated cattle with respect to their change in novel feed intake from the first to second day (Figure 5). Kronberg and Walker (unpublished data) observed that air-dried leafy spurge did not elicit a feed aversion in one of five yearling cattle

gavaged with air-dried leafy spurge after ingesting a novel feed. If some cattle do not experience aversions to leafy spurge after one exposure, then it is logical that not all cattle will experience an aversion from a single dose of ingenol that is similar to that in leafy spurge. The response of cattle to ingenol 3-benzoate was similar to that observed with rats (Kronberg et al., 1995). Rats formed an aversion to a novel fluid when they received a gastric infusion of ingenol 3-monobenzoate that followed their initial drink of the novel fluid (Kronberg et al., 1995).

This study helps define toxic and aversive compounds in leafy spurge that are probably, at least in part, responsible for the toxic and aversive effect that leafy spurge has on cattle. In view of the widespread distribution of leafy spurge on the northern Great Plains and its negative impact on cattle production in the region, understanding the chemical interaction between cattle and leafy spurge is important. Furthermore, given that sheep and goats will ingest substantial amounts of leafy spurge and that cattle may ingest small amounts, it is of considerable importance to elucidate the overall environmental risk factors for the potential cumulative toxicity of these cocarcinogenic compounds. Several reports have suggested that direct or indirect exposure of people and livestock to such plants or plant parts may lead to a potential risk of cancer by conditional carcinogenesis (Upadhyay et al., 1978; Hecker, 1981, 1987a,b; Zayed et al., 1998; Vogg et al., 1999). This clearly indicates the necessity to investigate the overall toxicity of leafy spurge on livestock, especially in areas highly infested with this noxius weed.

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GEOPHAGY AS A CORRELATE OF FOLIVORY IN RED-HANDED HOWLER MONKEYS (*Alouatta belzebul*) FROM EASTERN BRAZILIAN AMAZONIA

LUCIANE L. DE SOUZA,[1,*] STEPHEN F. FERRARI,[1,2] MARCONDES L. DA COSTA,[3] and DIRSE C. KERN[4]

[1]*Department of Zoology*

[2]*Department of Genetics*

[3]*Museum of Geosciences*
Universidade Federal do Pará
66.075-900 Belém—PA, Brazil

[4]*Department of Ecology*
Museu Paraense Emílio Goeldi
Caixa Postal 399, 66.017-970 Belém—PA, Brazil

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Abstract—Howler monkeys, *Alouatta* spp., are the most folivorous of neotropical primates (Platyrrhini), although Amazonian red-handed howlers (*Alouatta belzebul*) are relatively frugivorous. The feeding ecology of a free-ranging group of *A. belzebul* was monitored at a site in eastern Brazilian Amazonia over a ten-month period (November 1997 to August 1998). The first half of the study period encompassed the peak of the wet season, during which the study group's diet was predominantly frugivorous (scan sample records: 53.5% fruit, 40.8% leaves), and the second half, the transition from wet to dry season, characterized by a marked shift to folivory (18.9% fruit, 77.9% leaves). This shift was accompanied by a marked increase in mature, as opposed to flush leaves, which are relatively rich in secondary compounds. Ingestion of soil from termitaria was recorded on a total of 26 occasions, all of which occurred during the second half of the study period. Soil from termitaria was relatively rich in elements such as Ca and Na and in organic carbon, in comparison with that from the forest floor. The extent to which the monkeys ingested soil for their mineral supplements, or as an aid for the digestion of leaves, in particular the absorption of secondary compounds, remains unclear. The marked correlation with the observed patterns of folivory

* To whom correspondence should be addressed. E-mail: llopesouza@zipmail.com.br

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suggests that the latter function may have been the primary motive for geophagy in this species.

Key Words—Geophagy, soil geochemistry, howler monkey, *Alouatta belzebul*, folivory, Amazonia, detoxification.

INTRODUCTION

The systematic ingestion of soil has been observed in a wide range of vertebrates, including a number of primate species. Among primates, geophagy is apparently most common in arboreal folivores such as the Old World colobines (*Colobus*: Oates, 1978; *Presbytis*, Davies and Baillie, 1988), and the New World genus *Alouatta* (Hirabuki and Izawa, 1990; Izawa, 1993; Julliot and Sabatier, 1993; Bicca-Marques and Calegaro-Marques, 1994). Geophagy has been recorded in neotropical primates as diverse ecologically as spider monkeys (*Ateles*: Izawa, 1993), sakis (*Pithecia*: Setz et al., 1999), bearded sakis (*Chiropotes*: Ferrari, 1995), titi monkeys (*Callicebus*: Müller et al., 1997), and tamarins (*Saguinus*: Heymann and Hartmann, 1991), but appears to be less frequent overall than in howler monkeys, *Alouatta*.

Howler monkeys are the most widely distributed of platyrrhine (New World) primates, ranging from northern Argentina to southern Mexico, and are the best studied in the wild (Milton, 1980; Neville et al., 1988), although surprisingly little is known of the ecology of Amazonian populations (*Alouatta belzebul* and *Alouatta seniculus*). The data that are available (Julliot and Sabatier, 1993; Queiroz, 1995; Jardim, 1997) indicate that Amazonian howlers may be more frugivorous than those in other biomes, although folivory is seasonally important, as shown here.

The primary function of geophagy is thought to be the provision of mineral supplements, in particular such elements as sodium and calcium (Davies and Baillie, 1988; Hirabuki and Izawa, 1990; Mahaney et al., 1999). Soil may also be an important digestive aid for folivores, through the absorption of toxins, the balancing of gastric acidity (Oates, 1978; Davies and Baillie, 1988; Johns and Duquette, 1991; Aufreiter et al., 2001), and for its clay content (Ketch et al., 2001). Secondary compounds such as tannins, alkaloids, and terpenoids are especially abundant in mature leaves (Feeny, 1992). One alternative interpretation is that ingested soil helps combat endoparasite infestation (Bicca-Marques and Calegaro-Marques, 1994; Knezevich, 1995; Mahaney et al., 1997).

In the present study, data are presented on the occurrence of geophagy in free-ranging *A. belzebul* at a site in eastern Brazilian Amazonia, the first data on this type of behavior in this species. Ingestion of soil appears to be a relatively frequent activity, although it was confined to the second half of the study period, during which the proportion of leaves in the study group's diet practically doubled.

While geophagy is clearly correlated with the occurrence of folivory, it remains unclear whether and to what extent it provided mineral supplements or aided in the digestion of leaves.

METHODS AND MATERIALS

The 33,000-ha Ferreira Penna Scientific Station (*Estação Científica Ferreira Penna*, ECFPn) is located in the northernmost extreme of the Caxiuanã National Forest (1°41′S, 50°29′W), in the Brazilian state of Pará, eastern Amazonia. The research station is covered almost entirely by primary lowland Amazonian *terra firme* forest, bordered by blackwater *igapó* swamps (Almeida et al., 1993). Annual precipitation is between 2000 and 2500 mm, with a marked rainy season between December and May.

The present study focused on a social group of *A. belzebul* (denominated group "L" in Souza, 1999, and Souza et al., 2002) with nine members, one adult male, three adult females, one subadult male, two subadult females, and two infants. The group was monitored and habituated to the presence of human observers between July and October 1997, and the behavior of study group members was monitored systematically from November 1997 to August 1998. For the purposes of the present study, this period was divided into two five-month sessions, referred to here as the "wet season" (November to March) and the "dry season" (April to August).

Quantitative behavioral data were collected in scan samples, following procedures adapted from previous studies of *Alouatta* (Bonvicino, 1989; Mendes, 1989; Queiroz, 1995; Jardim, 1997). Five-minute scans of the study group were conducted at 15-min intervals throughout the diurnal cycle (06:00–19:00 hr) on three to five days each month. During each scan, the activity state of each visible group member was recorded at the moment of sighting. Only records of feeding behavior are considered here. Whenever the subject was observed ingesting an item of food, its characteristics were recorded (e.g., ripe fruit, flush leaf, flower bud), and the source was marked for taxonomic identification (see Souza, 1999; Souza et al., 2002). In addition, behavioral sampling was used for the recording of all observed occurrences of specific behavior categories, including geophagy. On each occasion that a group member was observed ingesting soil during the observation period, the source, location and individual(s) involved were recorded, as was the duration of soil ingestion.

Samples of approximately 1000 g of soil were collected from three of the termitaria exploited by study group members and from the ground in proximity to them and sent directly to the laboratory for drying and analysis. Termites found in three of the nests were collected in 70°C ethanol and deposited at the Zoology Department of the Goeldi Museum for identification.

The mineral content of the soil sample was analyzed using X-ray diffraction (XRD) on a Philips PW1050 diffractometer using a PW3710 control system. The XRD diagrams were obtained under the following equipmental conditions: copper anode, tension of 45 kV, electrical current of 40 mA at an interval of 4 to 60° 2θ. The mineral quantification was carried out through stoichiometric calculations using the chemical data of whole sample material. The chemical composition of the whole sample was defined using atomic absorption spectrometry, calorimetry, and titrimetry. Organic carbon was analyzed by using a modified Walkley-Black method (EMBRAPA, 1979), and determined by titration and trituration.

RESULTS

Overall, study group members fed primarily on leaves (60.54% of feeding records, with identified items), with the remainder of their diet being composed of fruit (35.08%) and flowers (4.38%). The relative contribution of leaves and fruit to the study group's diet changed drastically between the two seasons (Table 1). There was a similar shift in the consumption of leaves of different maturity. In the wet season, a majority (51.3%) of the leaves ingested were mature, whereas in the dry season, their contribution to the consumption of vegetative plant parts fell to only 4.0%.

Ingestion of soil from termitaria by study group members was not observed during wet-season months, but was recorded on eight of the 24 complete observation days carried out between April and August (12 scan records, 14 behavioral records). Observation time—number of scan samples—was only slightly (8.4%) longer during the dry season, so the marked seasonal contrast in the frequency of geophagy cannot be accounted for by differences in sampling effort. Despite the clear seasonal pattern, the temporal distribution of geophagy bouts was highly irregular. The group was observed returning to the same termitarium on some consecutive days, but also apparently ignored termitaria on other days, even when passing through the same tree crowns in which they were located.

TABLE 1. SEASONAL VARIATION IN DIET OF *A. belzebul* STUDY GROUP AT ECFPn

Item	Number of records (% of total)	
	Wet season	Dry season
Fruit	177 (53.5)	71 (18.9)
Leaves	135 (40.8)	293 (77.9)
Flower	19 (5.7)	12 (3.2)
Records (scan samples)	331 (1079)	376 (1170)

Three different termitaria were exploited, all located at heights of over 10 m from the ground. Termites collected from three termitaria belonged to the family Termitidae, although only one sample was identified to species—*Labiotermes labralis*. Geophagy was typically initiated by the alpha male, followed by the females and their young. On most occasions, at least three group members ingested soil from the termitarium sequentially, extracting it with their hands or directly with their mouths. The behavior was invariably brief, and mean bout duration (time between the initiation of geophagy by a group member and the ingestion of the last piece of soil by this or another individual) was 3:44 ± 1:29 min ($N = 11$). Overall, the sum of the evidence, in particular the relatively short duration of bouts, indicates clearly that the ingestion of soil, rather than termites, was the primary objective of the ingestion of material from the termitaria. Fragments of termites were found in only one of the total of 51 fecal samples collected during the study.

Soil from the study area is composed primarily of kaolinite, quartz, anatase, and amorphic iron compounds (Kern and Costa, 1997), and the samples collected here exhibited similar characteristics (Table 2). Soil and termitaria samples at the same sites were also similar in composition, except that the organic content of termitaria was three to four times that of the soil. Samples become generally more similar when mass is balanced by excluding the organic matter, although the iron mineral appears to be enriched. As might be expected, the chemical composition of different samples is also similar (Table 3). There is little doubt, however, that the termitaria were constructed from local soils.

While the main chemical elements are similar in samples from the same site, Na, K, Mg, and Ca are concentrated between two- and eightfold in termitaria (with

TABLE 2. MINERAL COMPOSITION OF TERMITARIA AND NEARBY SOILS[a]

	Mineral (wt%)							
Site	Kaolinite	Quartz	Anatase	Goethite	Organic matter	Total	Organic carbon	Organic carbon ratio T/S
S1	37.0	50.6	1.9	4.6	3.1	97.2	2.0	
T1	35.5 (37.6)	46.4 (49.1)[b]	1.7 (1.8)	5.3 (5.6)	9.8	98.8	5.8	2.9
S2	35.8	53.1	1.9	5.4	3.9	100.0	2.1	
T2	34.1 (40.4)	39.8 (47.1)	1.1 (1.3)	6.2 (7.3)	16.6	97.9	8.1	3.9
S3	43.3	43.1	1.1	6.2	5.2	99.0	2.9	2.8
T3	35.8 (44.1)	31.8 (39.2)	0.9 (1.1)	7.5 (9.2)	23.0	98.7	8.1	

[a] S = Soil samples, T = termitaria. Samples from the same site have the same number.
[b] Values in parentheses are mass balanced for organic material.

TABLE 3. CHEMICAL COMPOSITION OF TERMITE HOUSING STRUCTURE AND NEARBY SOILS[a]

Chemical	Sample					
	S1	T1	S2	T2	S3	T3
SiO_2 (wt%)	67.83	62.87 (66.75)	69.72	55.67 (66.56)	63.21	48.17 (59.89)
Al_2O_3 (wt%)	14.60	14.01 (14.87)	14.13	13.49 (16.13)	17.10	14.16 (17.60)
TiO_2 (wt%)	1.93	1.67 (1.77)	1.95	1.08 (1.29)	1.12	0.92 (1.11)
Fe_2O_3tot (wt%)	4.14	4.80	4.87	5.59	5.62	6.77
NA (ppm)	447.5	865.8	636.5	663.8	348.5	834.6
K (ppm)	166.0	482.9	176.1	332.6	150.9	309.2
Mg (ppm)	225.2	380.0	240.1	476.0	234.4	586.8
Ca (ppm)	69.0	535.6	182.1	883.0	134.5	778.1
Mn (ppm)	222	226	173	221	187	162
P_2O_5 (wt%)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
PF (wt%)	8.71	15.19	9.41	21.89	11.78	28.75
Organic Carbon (wt %)	2.00	5.79	2.11	8.06	2.93	8.14

[a] Values in parentheses are mass balanced for organic material.

one exception—Na at site 2) when compared to local soil (Table 4). It seems likely that this concentration of alkaline and calkaline elements is a by-product of the introduction of organic matter by the termites during nest building. It is difficult to say to what extent the howlers' exploitation of soil from termitaria—rather than the forest floor—might have been motivated by such differences, given their highly arboreal habits (study group members were never observed in the canopy at heights of less than 6 m above the ground).

DISCUSSION

Geophagy appears to be relatively common in howler monkeys compared to other platyrrhines. However, the present study represents the first record of geophagy for *A. belzebul*, despite the fact that the feeding ecology of this species has been studied in detail in both Atlantic Forest (Bonvicino, 1989) and Amazonian (Jardim and Oliveira, 1997) biomes. The latter study was also conducted at the

TABLE 4. RATIO OF ELEMENTS IN TERMITARIA/SOIL

Site	Na	K	Mg	Ca	Mn
1	1.9	2.9	1.7	7.8	1.0
2	1.0	1.9	2.0	4.8	1.3
3	2.4	2.0	2.5	5.8	0.9

ECFPn, although it focused on a different study group (denominated "M" in Souza et al., 2002). The lack of records in both these studies reinforces the correlation with folivory found in the present study, given that the study animals were relatively frugivorous in both cases. Group M was, in fact, monitored during the equivalent of the wet-season period as defined here, during which the proportion of fruit in its diet was even larger than that recorded for group L (Jardim and Oliveira, 1997).

Members of the third group ("A" in Souza et al., 2002) that was monitored at the site during the same period as the present study were observed ingesting soil on three occasions, all during the dry season. It remains unclear, however, whether this relatively small number of records was the result of a real difference in the frequency of geophagy in this group or a consequence of sampling factors, given the irregularity of the behavior, as observed in group L. Certainly, it does not appear to be related to differences in the diet of group A, which was at least as folivorous as group L, especially during the dry season. More recent studies of *A. belzebul* at sites in eastern (A. Pinto, personal communication) and central Amazonia (Souza, personal observation) indicate that geophagy is widespread in Amazonian populations of this species.

Curiously, Izawa (1993) reported that *A. seniculus* apparently avoided termitaria made by the species recorded here, *Labiotermes labralis*, while preferring those made by species of the genus *Constrictotermes*. Exploitation of termitaria of the latter genus was also recorded for *A. seniculus* by Julliot and Sabatier (1993). Izawa (1993) suggested that the howler monkeys' apparent preference may have been related to termite predator avoidance strategies rather than the composition of its termitaria, although ingestion of soil by *A. belzebul* from the termitaria of *L. labralis* in the present study appears to contradict this.

Overall, the occurrence of geophagy during the dry-season months, when the consumption of leaves increased considerably, suggests that the primary function of this behavior may be as a digestive aid, as suggested in studies of other folivorous primates (Oates, 1978; Julliot and Sabatier, 1993; Milton, 1993). Plant chemical defenses are composed of secondary metabolites such as tannins, alkaloids, and terpenoids and are most abundant in immature fruits and mature leaves (Feeny, 1992). These compounds have an unpleasant taste and can be lethal to folivores (Milton, 1993). As observed in other mammals (Klaus et al., 1998), the high clay content and organic matter found in the soil ingested in the present study may help to absorb these compounds. Of course, the mineral content of the ingested soil cannot be ignored (Table 4; Davies and Baillie, 1988; Hirabuki and Izawa, 1990), but in the absence of data on the composition of fruit and leaves consumed during different seasons, it would be premature to conclude that mineral supplementation may have been the primary motivation for the ingestion of soil. Obviously, more detailed information on the chemical composition of the food items exploited by the howler monkeys would permit a more conclusive interpretation of the functional aspects of geophagy in this species.

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OCCURRENCE OF *p*-NONYLPHENOL ISOMERS IN WILD SPECIES OF *Cichorium endivia* subsp. *divaricatum*[1]

VALERY M. DEMBITSKY,[2,4,*] PAULINA GOLDSHLAG,[3] and MORRIS SREBNIK[2,4]

[2]*Department of Pharmaceutical Chemistry & Natural Products*
School of Pharmacy, P.O. Box 12065
The Hebrew University of Jerusalem
Jerusalem 91120, Israel

[3]*Plant Protection & Inspection Service*
Pesticide Residues Laboratory
Ministry of Agriculture
P.O. Box 78, Beth Dagan 50250, Israel

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Abstract—*p*-Nonylphenol isomers and other aromatic compounds were found in roots and stalks of the plant *Cichorium endivia* subsp. *divaricatum*. These compounds were characterized by high-resolution capillary GC-MS.

Key Words—*Cichorium*, *p*-nonylphenol isomers, medicinal herb, GC-MS.

INTRODUCTION

Alkylphenol ethoxylates belong to the group of nonionic surfactants (Dodge, 1998) and, in particular, nonylphenol ethoxylates have found use in the chemical manufacturing industry, in cosmetics, and as agrochemicals in different countries (Bennie, 1999). Nonylphenols are found in high concentrations in some sewage sludges that may be spread on agricultural lands (Bennie, 1999). Sewage treatment plants discharge significant amounts of nonylphenol ethoxylate degradation products in their final effluents and digested sludges compared to what enters the plant (Maguire, 1999). Alkylphenols and their ethoxylates also are found in sediments, river estuaries (Björn et al., 1997), and in marine waters of the Israeli Mediterranean coast (Zoller and Hushan, 2000).

* To whom correspondence should be addressed. E-mail: dvalery@cc.huji.ac.il
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[4] Affiliated with the David R. Bloom Centre for Pharmacy at the Hebrew University.

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Nonionic surfactants are extremely toxic to aquatic organisms and accumulate in macrophytic algae, particularly *Cladophora glomerata* (Ahel et al., 1993), other freshwater organisms (Ahel et al., 1993; Servos, 1999) that can then be eaten by fish, shellfish (Bennie, 1999; Tsuda et al., 2000), and mollusks (Björn et al., 1997). Traces of some nonionic surfactants are also found in different tissues of a wild duck (Ahel et al., 1993). Alkylphenols have been detected in 190 food samples purchased from markets, including fish, meat, and vegetables (Sasaki et al., 1999).

This report describes the composition of *p*-nonylphenols that were identified by gas chromatography–mass spectrometry (GC-MS) from wild species of *Cichorium endivia* subsp. *divaricatum* (Asteraceae).

METHODS AND MATERIALS

Plant Material. Cichorium endivia L. subsp. *divaricatum* (Schousb.) P. D. Sell was collected at Sdot Micha place (November 1999), about 25 km west of Jerusalem. A voucher specimen is kept at the Natural Products Department, School of Pharmacy. The plant was collected and identified by Prof. A. Danin (Institute of Life Science, Hebrew University).

Extraction Procedure. Dried stalks and roots of *C. endivia* subsp. *divaricatum*, were separately homogenized in a high-speed unit, and successively percolated for 2 hr each with pentane, CH_2Cl_2, and 10% NaOH in MeOH at 60°C. The aqueous phase was acidified and volatile compounds were extracted with pentane and CH_2Cl_2. Extracts were combined and the solvent was removed under reduced pressure. The oil sample (100 mg) was dissolved in toluene, and 1% sulfuric acid in methanol was added. The mixture was left overnight in a stoppered tube at 50°C, then, water (10 ml) was added. Volatile compounds were extracted with pentane and then CH_2Cl_2. The solution was filtered and solvent removed under reduced pressure. The oil residue was dissolved in $CH_2Cl_2$and stored at −20°C prior to GC-MS analysis.

GC-MS Analysis. GC-MS was performed with a Hewlett Packard 6890 series II chromatograph linked to a HP 5973 mass detector equipped with an HP automatic injector and a 30-m long, 0.25-mm ID, 0.25-μm film thickness HP-5MS capillary column. The ionization energy (EI) was 70 eV, the carrier gas was He (flow 1.0 ml/min), and the injection block temperature was 250°C. The GC oven temperature was programmed as follows: initial temperature 100°C (2 min) followed by a temperature increase of 15°C/min up to 260°C (10 min) and then 20°C/min to the final temperature of 290°C (10 min). Identification of the compounds was established using the synthetic *p*-nonylphenols (obtained from Sigma-Israel and ChemTech., Ltd, Israel), and also the Wiley 7th Edition Library.

RESULTS AND DISCUSSION

Analysis of the total oil extracts from the stalks of a wild population of *C. endivia* subsp. *divaricatum*, collected in a dry state in November 1999, showed that they contain *p*-nonylphenol isomers and some other aromatic compounds. In order to unequivocally characterize the *p*-nonylphenol isomers and other aromatics in the mixture, it was necessary to achieve a good separation of all isomers of *p*-nonylphenols present in the natural extract. Figure 1B shows the gas chromatogram of the *p*-nonylphenol isomeric mixture and other aromatics. Concentrations of *p*-nonylphenols were found to be 138.6 and 23.4 μg/kg in dried roots and stalks, respectively.

There are several mass spectra of *p*-substituted alkylphenols that can be examined in order to provide insight to unknown *p*-nonylphenol isomers. All *p*-alkylated phenols produce a molecular ion in the EI mode followed by cleavage of the alkyl group. Mass spectra can be grouped into three categories with respect to the α-carbon on the alkyl chain: primary ($-CH_3-$), secondary ($-CHR_1-$), and tertiary ($-CR_1R_2-$). The GC-MS ion chromatogram includes seven separated characterized ions (*m*/*z*): 107, 121, 135, 149, 163, 177, 191, and also the molecular ion 220, which is common for all *p*-nonylphenol isomers (Figure 2). Primary α-carbon *p*-nonylphenols form the base peak fragment ion *m*/*z* 107 via simple benzylic cleavage. The fragment ions of *m*/*z* 107 and 121 are also common to all *p*-nonylphenols (peaks 5–16) (Figure 2). Secondary α-carbon *p*-nonylphenols, with one hydrogen attached to the benzylic carbon, fragment to the α-methyl carbocation *m*/*z* 121. Tertiary α-carbon compounds produce spectra with *m*/*z* 135 as the base peak. The dimethyl carbocation is stable and, therefore, its formation is energetically favorable. The propyl fragment results in a large abundance of *m*/*z* 149 ions. Presence of the base peak of *m*/*z* 163 can be interpreted by loss of a butyl fragment from the β-carbon.

Several papers deal with HPLC (Björn et al., 1997; Tsuda et al., 2000) or GC-MS analysis of *p*-nonylphenol isomers and their ethoxylates (Bhatt et al., 1992; Wheeler et al., 1997; Hao et al., 2000; Nakamura et al., 2000). For identification of all *p*-nonylphenol isomers, we used mass libraries and also the mass spectra of model alkylphenols as reported by Bhatt et al. (1992) and Wheeler et al. (1997).

Occurrence of different alkylphenol types isolated from plants has been reported (Tyman, 1991; Kozubek and Tyman, 1999). Alkenyl phenols with short-chains (C_8 and C_{10}) were isolated from the medicinal plant *Piper gibbilimbum* (Piperaceae; Papua New Guinea) (Orjala et al., 1998). The phenolic lipids with long-chains (C_{11}–C_{15}) were isolated from the liverwort *Schistochila appendiculata* (Asakawa et al., 1987) and identified from the oil of *Anacardium occidentale* (Anacardiaceae) (Tyman, 1991).

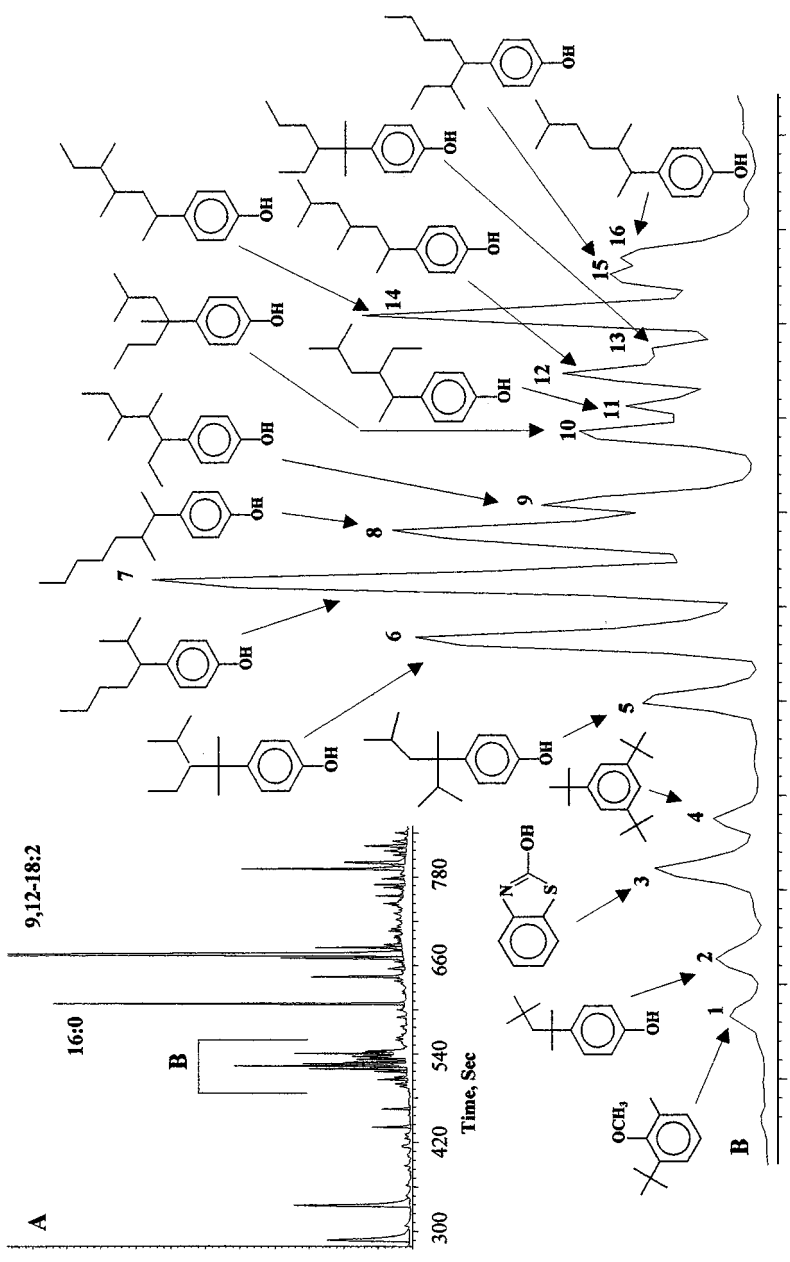


FIG. 1. GC-MS chromatogram of total extract from the stalks of *Cichorium endivia*. (A) Full run 2000 sec. Major components: hexadecanoic acid, methyl ester (16:0, 7%) and octadecadienoic acid, methyl ester (9,12–18:2, 38.5%). (B) Part of the gas chromatogram of the *p*-nonylphenol isomers on a cross-linked HP-5MS 30-m × 0.25-mm capillary column.

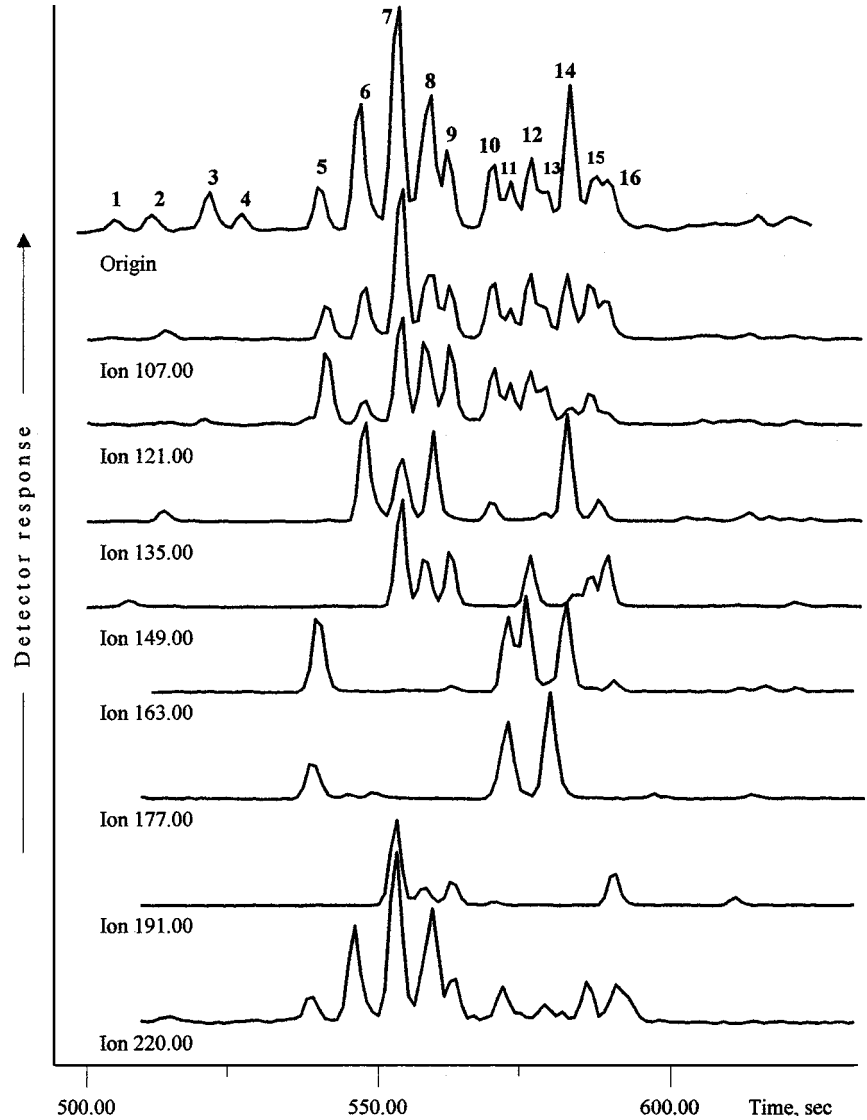


FIG. 2. GC-MS selected ion monitoring chromatograms illustrating *p*-nonylphenol isomer coelution. Peaks numbers and structures refer to Figure 1.

The goal of the work described in the present paper was to identify *p*-nonylphenol isomers and other aromatics in the wild medicinal plant *Cichorium endivia* subsp. *divaricatum*. We do not know the origin of nonylphenols in the roots and stalks of this plant; however, it is reasonable to assume that the phenols

can accumulate from groundwater. Checking this hypothesis requires additional studies.

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DEFENSIVE SECRETION OF *Therea petiveriana*: CHEMICAL IDENTIFICATION AND EVIDENCE OF AN ALARM FUNCTION

JEAN-PIERRE FARINE,[1,*] ETIENNE SEMON,[2] CLAUDE EVERAERTS,[1] DEHBIA ABED,[1] PHILIPPE GRANDCOLAS,[3] and REMY BROSSUT[1]

[1]*Université de Bourgogne, CNRS, UMR 5548*
6 Bd Gabriel, 21000, Dijon, France

[2]*INRA, Laboratoire de Recherches sur les Arômes*
17 Rue Sully, 21034, Dijon, France

[3]*ESA 8043, CNRS, Laboratoire d'Entomologie*
Muséum National d'Histoire Naturelle
45 Rue Buffon, 75005, Paris, France

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Abstract—The volatile constituents of the supposed defensive secretions of the glandular pouches of the adults of both sexes of the cockroach *Therea petiveriana* have been shown to contain *N*-3-methylbutylacetamide (MBA) and *N*-3-methylbutylpropanamide (MBP), which represented 60% of the volatile fraction. The other 40% included acidic, aromatic, and aldehydic compounds. Behavioral experiments demonstrated that the secretion acts as an alarm pheromone for adults.

Key Words—Dictyoptera, Polyphagidae, *Therea petiveriana*, exocrine glands, pheromones, alarm.

INTRODUCTION

Therea petiveriana (Dictyoptera, Polyphagidae) belongs to a basal family of cockroaches (Grandcolas, 1993, 1997) and was reported from peninsular India (Princis, 1964). It is a fossorial species, inhabiting rubbish heaps around human habitations as well as in humus accumulations in certain isolated pockets of the scrub jungles and semiarid zones of south India. The adults exhibit a strong tendency towards gregarious activities. They are essentially crepuscular, and during nonactive

*To whom correspondence should be addressed. E-mail: Jean-Pierre.Farine@u-bourgogne.fr

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periods they remain subterranean. Adult males and females are macropterous, but flightless. When disturbed, they raise their wings and evert on either side, on the second and third abdominal segments, a pair of brown pouches (Gerstaecker, 1861; Livingstone and Ramani, 1978; Brossut and Sreng, 1985). These organs, described in various species of Polyphagidae (Grandcolas, 1997), are absent in the nymphal instars. Normally, the pouches are never visible and are withdrawn into the body cavity. The exposed surface of the pouch reveals numerous chitinous pleats of varying heights, which bear whorls of fine setae that are generally enveloped with secretion (Livingstone and Ramani, 1978).

In several families of insects, the functions of medium-sized exocrine glands producing complex mixtures have been well investigated. On the other hand, most of the very large exocrine glands were "so obviously" defensive (Roth and Alsop, 1978) that few investigations have been conducted on their other possible functions. Since the preliminary observations of Livingstone and Ramani (1978) on *T. petiveriana* suggested a defensive or alerting function of the pouch, we undertook a chemical analysis of the pouch glandular secretions. The alarm role of the whole secretion and of its two major identified components were investigated.

METHODS AND MATERIALS

Insects. Colonies of *T. petiveriana*, obtained from the Laboratoire de Primatologie (CNRS UA 373, Station biologique de Paimpont, France), were maintained in glass aquaria (60 × 40 × 20 cm) at 28–30°C, 80% relative humidity, and 12L:12D photoregime. The insects had free access to dry dog food and water-soaked cotton pads. It was difficult to maintain this species under laboratory conditions, and it was impossible to breed large colonies.

Preparation of Extracts. Adults of both sexes (30 to 40 days old) were cooled for a few minutes at −20°C before use. The four glandular pouchs were artificially everted using light pressure on the abdomen with forceps. Then, the glands of one individual were cut using microscissors and pooled in a vial containing 100 μl of distilled methylene chloride (CH_2Cl_2) and 500 ng of 2-phenylethanol as internal standard. The pieces were allowed to soak for 15 min at room temperature. Then the extracts were filtered through glasswool and kept at −20°C until analyzed.

Chemical Analysis. GC analyses of extracts were performed using a Packard 437A chromatograph fitted with a flame-ionization detector. CP Wax 58 CB (30 m × 0.25 mm ID, 0.22-μm film thickness, Chrompack) and CP Sil 5 CB (30 m × 0.25 mm ID, 0.22-μm film thickness, Chrompack) fused silica capillary columns were used for analyses. After concentration of the extract to about 10 μl under gentle nitrogen flow, 2 μl of sample was injected into a split-splitless injection system, operating with a split flow of 25 ml/min and a septum purge of

2 ml/min. The split port was closed during injection and then opened for 30 sec after injection. The column was held isothermally at 40°C for 2 min, then programmed at a rate of 20°C/min for 1 min, 2°C/min to 150°C, and then 10°C/min to 250°C. Hydrogen was used as carrier gas (50 cm/sec velocity at room temperature). The injector and detector temperatures were 250 and 270°C, respectively. Collection of the data was done automatically by PC software (Maestro, Chrompack).

GC-MS was carried out using a Nermag R 10-10 C quadrupole mass spectrometer coupled with a HP 5890 gas chromatograph (Hewlett Packard). The GC conditions were the same as those just described. The column was connected directly to the ion source of the spectrometer through a heated transfer line maintained at 260°C. Electron impact (EI) mass spectra were obtained at 70 eV with a 0.8-sec cycle, the instrument scanning from 25 to 300 amu with a source temperature of 150°C. Chemical ionization (CI) spectra, using ammonia (NH_3) and deuterated ammonia (ND_3) with a source pressure of 0.3 torr, were obtained at 90 eV with a source temperature of 90°C, the instrument scanning from 60 to 300 amu in 0.7 sec.

Compounds were identified by comparing their spectra to those of the libraries of the EPA/NIH (Heller and Milne, 1978), of the NIST/EPA/MSDC (Lias and Stein, 1990), and of the Laboratoire de Recherches sur les Arômes (INRA, Dijon, France). Except for *N*-3-methylbutylpropanamide (MBP) (gift from Dr. R. Henry, INSA, Lyon, France), all other identified chemicals were purchased from Interchim (Montluçon, France). Identification of all the compounds was checked by analyzing the natural products and their synthetic counterparts under the same conditions on CP Sil 5 CB and CP Wax 58 CB columns. Numerous components of the secretion appeared in quantities of <1 ng/insect; consequently, the majority of them were not identified and quantified.

Fourier transform infrared (FTIR) spectra were taken in the gas phase with a Bruker IFS 85 FTIR spectrometer that was coupled, via a Bruker gold-coated light-pipe (20 cm $\times$ 0.8 mm ID) maintained at 220°C, to a Carlo-Erba 5160 gas chromatograph. The GC instrument was equipped with the same column as that already described, an on-column injector, and a flame ionization detector. Helium was used as the carrier gas (35 cm/sec velocity at 220°C), and the oven temperature was programmed from 40 to 220°C at 3°C/min. Nitrogen gas was introduced into the transfer line to reduce peak broadening within the light-pipe, resulting in a total gas flow rate of 5 ml/min. The spectral resolution was 8 cm^{-1}, and the time resolution was reduced from 12 collected interferograms per second to four effective ones by adding three interferograms in real time. A narrow-band (4800–600 cm^{-1}) mercury–cadmium–telluride detector was used.

Chemical ionization and FTIR analyses were used to characterize the two major components of the secretion (peaks 5 and 6). Quantitative analyses were made on five males and four females. The response factor was determined for each of the identified major compounds at 1, 10, 100, and 500 ng. These response factors were used for quantitative analyses.

Behavioral Observations. All the observations were performed in open plastic arenas (12 × 8 × 5 cm) at the same temperature and relative humidity as described above. The behavioral experiments were conducted between the second and the fourth hours of the scotophase using 30- to 40-day-old adults of both sexes.

In order to determine the role of the secretion at close-range on adults of both sexes, we tested the activity of various amounts (1, 10, 100, 500, and 1000 ng) of the major components MBA and MBP and compared their activity to the whole secretion. Because of the lack of insects to be tested, only one dosage of the whole secretion was investigated; this concentration corresponds to the secretion from one adult (1 eq/insect).

A small piece of filter paper (0.2 × 2 cm) was mounted on an iron stem. Preliminary tests confirmed that the device did not elicit any insect behavior. The paper was impregnated with the solution to be tested. After evaporation of the solvent (CH_2Cl_2), the paper was brought within 1 cm of the insect antennae and maintained for a maximun of 1 min. The reaction of the insect was noted and the corresponding latency period before a behavioral response recorded. Each experiment was replicated 10 times on different subjects and the data was compared by using a Mann-Whitney *U* test.

RESULTS

Chemical Identification. A typical GC trace derived from the pleural glandular secretions of adults of both sexes revealed the presence of a mixture of volatile compounds and long-retention-time (>70 min) constituents, with an estimated ratio of 25:75 (Figure 1). No qualitative difference appeared between males and females. The only quantitative differences between the secretions of both sexes were found in peaks 5, 6, and phenol.

Based on their MS spectra, the major long-retention-time compounds were identified as saturated or unsaturated fatty acids (e.g., C_{14}, C_{16}, C_{18}, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ acids). All these acids are known to be part of the insect fat body. The volatile fraction of the secretion showed the presence of about 40 volatile compounds (molecular weight from 60 to 200). The two major compounds (peaks 5 and 6) represent about 60% of this fraction. The rest of the secretion is a mixture of saturated acids (12%), aromatic (10%), and aldehydic (2%) compounds (Figure 1). Only six of the identified compounds appeared to be present at >1 ng/insect (Table 1).

Compound 5 was unambiguously identified as *N*-3-methylbutylacetamide (MBA), or *N*-isoamylacetamide, by comparison of its mass [electron impact (Figure 2a) and chemical ionization modes], and FTIR spectra (Figure 3a) and retention index with those of an authentic standard. Quantitative analyses revealed that MBA represented about 47% of the volatile fraction of the whole male secretion and about 30% of the volatile female fraction (Table 1).

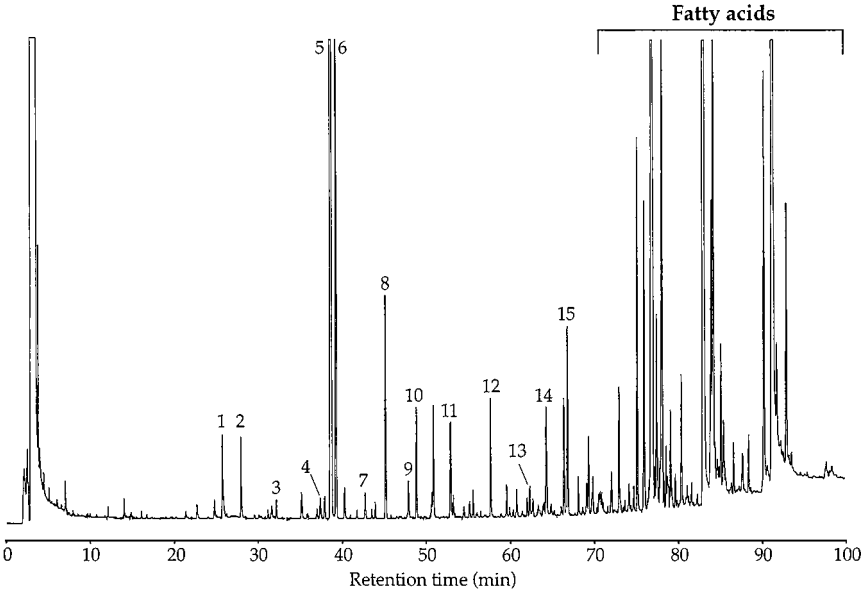


FIG. 1. Typical GC trace and identified compounds of the defensive secretion from male *T. petiveriana*: 1, nonanal; 2, acetic acid; 3, butanoic acid; 4, hexanoic acid; 5, *N*-3-methylbutylacetamide (MBA); 6, *N*-3-methylbutylpropanamide (MBP); 7, heptanoic acid; 8, phenol; 10, *p*-cresol; 11, nonanoic acid; 12, decanoic acid; 13, undecanoic acid; 14, indol; 15, dodecanoic acid.

TABLE 1. RELATIVE PROPORTIONS OF THE MAJOR IDENTIFIED COMPOUNDS OF DEFENSIVE SECRETIONS FROM *T. petiveriana* MALES AND FEMALES[a]

Compound	Proportion (ng, mean ± SEM)		
	Males ($N = 5$)	Females ($N = 4$)	P
Nonanal	2.80 ± 0.75	3.00 ± 0.71	0.695
MBA	244.80 ± 11.16	89.00 ± 10.42	0.014
MBP	13.40 ± 2.42	63.75 ± 7.92	0.014
Phenol	13.00 ± 1.10	16.50 ± 1.12	0.014
p-Cresol	14.16 ± 1.85	17.50 ± 1.66	0.083
Nonanoic Acid	6.00 ± 1.41	6.50 ± 1.12	0.618

[a] MBA, *N*-3-methylbutylacetamide; MBP, *N*-3-methylbutylpropanamide. *P*, Mann-Whitney *U* test.

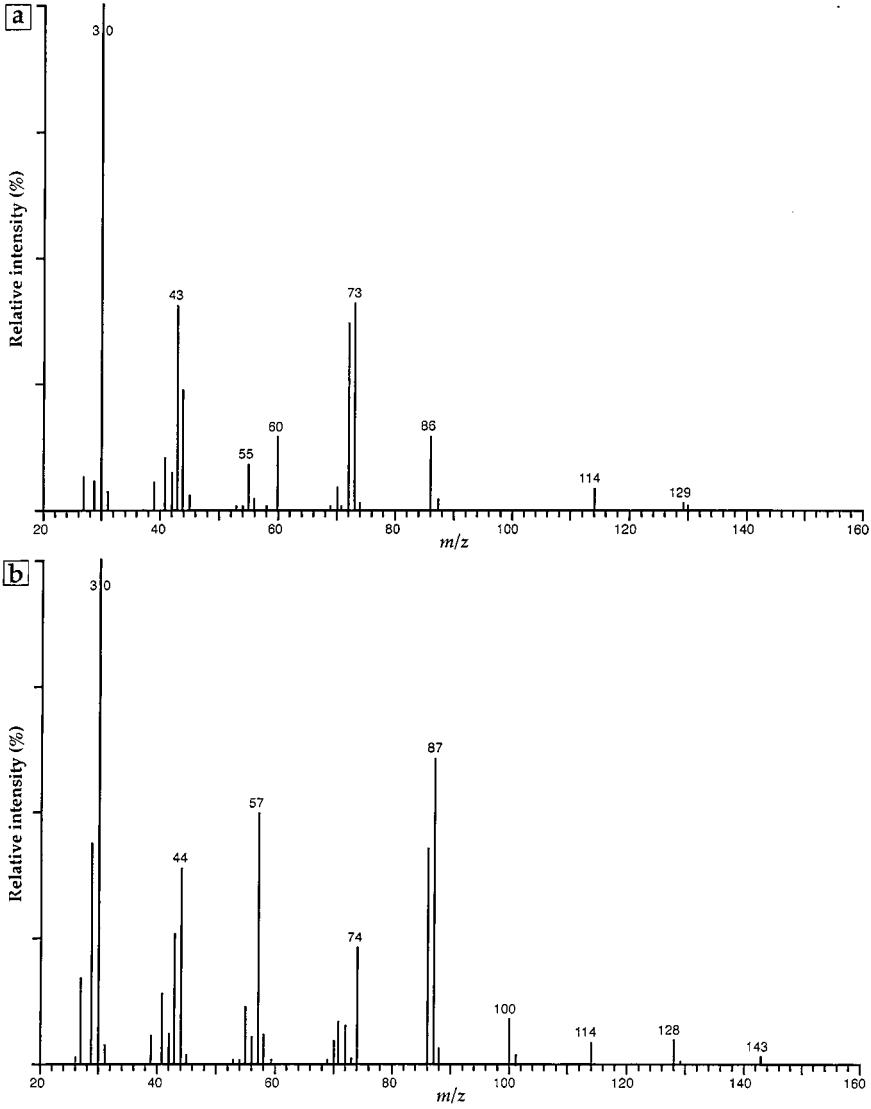


FIG. 2. Electron impact mass spectrum of peaks 5 (a) and 6 (b) identified as *N*-3-methylbutylacetamide (MBA) and *N*-3-methylbutylpropanamide (MBP), respectively.

The mass spectrum of compound 6 showed a parent peak of low intensity at *m/z* 143 (Figure 2b). This mass spectrum was not found in mass spectral data bases. Its molecular weight of 143 was confirmed by the observation of the ions *m/z* 144 $(M+H)^+$ and *m/z* 161 $(M+NH_4)^+$ in the CI (NH_3) mass spectrum. The

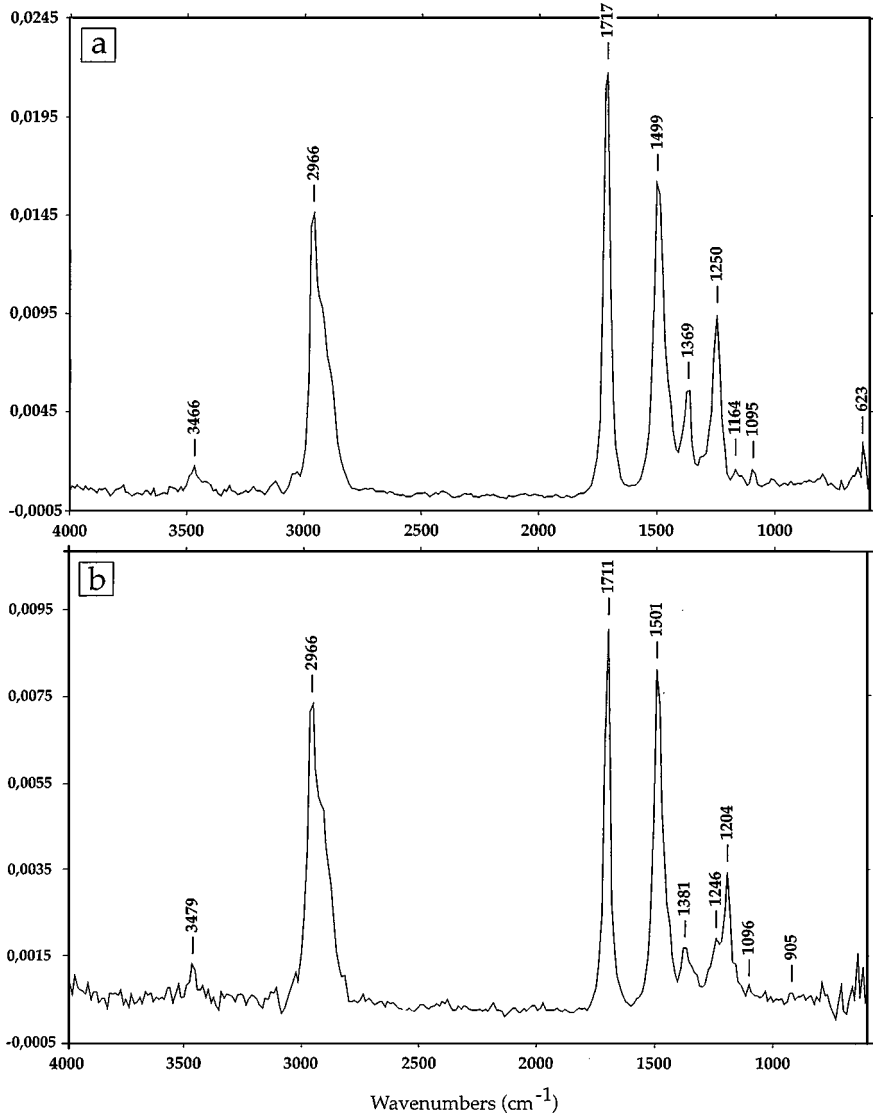


FIG. 3. GC infrared spectrum of peaks 5 (a) and 6 (b) identified as *N*-3-methylbutylacetamide (MBA) and *N*-3-methylbutylpropanamide (MBP), respectively.

exchange of one hydrogen for deuterium has been processed by deuterated ammonia chemical ionization. The CI (ND_3) spectrum showed a shift of the ions from *m*/*z* 144 to *m*/*z* 146 and from *m*/*z* 161 to *m*/*z* 166, corresponding to $(M–H+D_2)^+$ and $(M–H+D+ND_4)^+$, respectively. A similar shift was observed in the CI (ND_3)

TABLE 2. CLOSE-RANGE ACTIVITY OF VARIOUS AMOUNTS OF DEFENSIVE SECRETION COMPONENTS MBA AND MBP ON *T. petiveriana* ADULTS OF BOTH SEXES[a]

	Latency period (sec, mean ± SEM)					
	Males ($N = 10$)			Females ($N = 10$)		
Amount (ng)	MBA	MBP	*P*	MBA	MBP	*P*
10	29.90 ± 8.10	28.70 ± 5.60	0.649	28.20 ± 2.43	22.50 ± 4.52	0.011
100	13.30 ± 4.03	18.20 ± 2.44	0.011	19.60 ± 3.35	13.60 ± 2.73	0.002
500	3.50 ± 1.12	4.40 ± 1.11	0.001	3.60 ± 1.62	3.20 ± 1.66	0.538
1000	2.40 ± 1.43	2.70 ± 1.42	0.006	2.60 ± 1.02	2.70 ± 1.19	0.937

[a] Latency period of insects running away. MBA, *N*-3-methylbutylacetamide; MBP, *N*-3-methylbutylpropanamide. *P*, Mann-Whitney *U* test.

mass spectrum of isoamylacetamide. A comparison of the infrared spectra of MBA and of compound 6 (Figure 3b) supported the idea that these two compounds were analogs. Thus, the presence of the bands at 1711 cm^{-1} ($\nu_{C=O}$), 3479 cm^{-1} (ν_{N-H}) and 1501 cm^{-1} (ν_{N-H}) in the FTIR spectrum revealed an amide function (Nyquist, 1984).

All these observations led us to conclude that compound 6 was *N*-3-methylbutylpropanamide (MBP) or *N*-isoamylpropanamide. Coinjection with the authentic sample confirmed that the synthetic and isolated compounds were identical. In males, MBP represents about 16% of the volatile fraction, whereas its amount is higher in females (30% of the volatile fraction) (Table 1).

Close-Range Activity of Secretion. The biological activities of MBA and MBP showed that these two components were repellent to both sexes (Table 2). Males and females did not respond to 1 ng of the two tested compounds. For highest concentrations, all the individuals ran away from the chemical source in the same way; higher concentrations (10–1000 ng) elicited faster escape responses.

When the equivalent of one male glandular extract was tested, the reaction of both sexes was similar and all the tested individuals ran away from the chemical source immediately (2.10 ± 0.70 sec for males, 2.20 ± 0.75 sec for females). However, when the female glandular extract was presented, the tested females escaped more quickly ($P < 0.001$) than did the males (2.50 ± 0.81 sec for females; 6.40 ± 2.79 sec for males). Additionally, our data showed that females responded in the same way when they were confronted with male or female glandular extracts, whereas males were more repelled by male extracts. ($P < 0.001$)

DISCUSSION

Cockroaches possess a complex abdominal glandular exocrine system (Brossut and Roth, 1977; Brossut and Sreng, 1980). The identified pheromones

play different roles in the courtship and mating sequences (Nishida et al., 1974, 1976; Persoons et al., 1990; Sreng, 1990). Glandular defensive secretions occur in various species (Roth and Alsop, 1978; Brossut, 1983), especially in the Polyzosteriinae, in which ventral intersegmental defensive glands seem to be the rule (Stay, 1957; Chada et al., 1961; Waterhouse and Wallbank, 1967; Brossut and Sreng, 1980). The defensive glands are located on the pleura, abdominal spiracles, or abdominal interstermites or intertergites. Allomones are especially efficient at deterring some nonswarming predators (Eisner et al., 1959).

In many species of cockroaches, habitat selection is different for nymphs and sexually mature adults (Schal et al., 1984). Most nymphs live on the ground, where shelters are abundant, while adults, who are often exposed to many potential predators while searching for mates, need more efficient defenses. Nymphs and adults of the Blattidae species have evolved specific defensive strategies. Nymphs depend on the secretion of deterrent glues produced by abdominal segments (Ichinosé and Zennyoji, 1980; Schal et al., 1982; Abed et al., 1994), while adults spray the secretion of their large defensive sternal glands. The burrowing nymphs of *T. petiveriana* never revealed the presence of glues on nymphs (Farine, personal observations), whereas defensive glands were present in adults.

The diversity of compounds that have been identified as exocrine products in arthropods has emphasized the biosynthetic versatility that these invertebrates possess. The products of exocrine glands almost invariably consist of mixtures of compounds, and the roles of minor constituents in augmenting the value of defensive exudates have been well established.

Our chemical data revealed that, besides the two major identified amides, which represent 60% of the volatile defensive components of *T. petiveriana*, about 25% of the secretion was composed of saturated acids, as well as aromatic and aldehydic chemicals. References to the defensive secretions of Dictyoptera, Hymenoptera, Coleoptera, and Hymenoptera (Tschinkel, 1975; Klinger and Maschwitz, 1977; Staddon, 1979; Blum, 1981; Dettner, 1987; Aldrich, 1988; Morgan and Mandava, 1988) have shown that a number of acidic, aldehydic, and aromatic compounds reported in this study appeared as deterrents for either invertebrate or vertebrate predators (Blum, 1981, and references therein). A blend of many compounds of various chemical families could thus increase the potency of the defensive secretion of *T. petiveriana*, especially against ants, which are known to be common predators of numerous insects.

Simple amides such as MBA have long been identified in California sherry (Webb et al., 1966), tobacco (Demole and Berthet, 1972), and wines (Schneider et al., 1998; Miklosy et al., 2000). In insects, the two identified amides in *T. petiveriana*, MBA and MBP, were first reported in the rectal glandular secretion of males of various species of fruit flies (Metcalf, 1990, and references therein) and from the venom of wasps (Heath and Landolt, 1988; Weston et al., 1997). In the Queensland fruit flies *Dacus tryoni* and *D. neohumeralis*, sexually

excited males emit an oily secretion with a sweet pungent odor, that is distributed by wing fanning; this attracts the specific females over a range of several meters. The source of the secretion is a glandular complex that opens into the rectum (Fletcher, 1969; Schultz and Bousch, 1971). In these two species, the secretion was identified as a mixture of six aliphatic amides in which MBA and MBP appeared as major compounds (Bellas and Fletcher, 1979). In *D. dorsalis*, MBA elicited zig-zag anemotactic flight in mature and virgin females (Kobayashi et al., 1978). In *Vespula squamosa*, *N*-isopentylacetamide, or MBA, was described as a pheromone that elicits alarm and attack behavior (Heath and Landolt, 1988). However, the same component attracts the workers of *V. vulgaris* when used at low concentration, whereas larger amounts were repellent (Weston et al., 1997).

Our behavioral observations showed that the adults of *T. petiveriana* escaped when low and large concentrations of MBA and MBP were used alone. However, when the whole secretion of female was tested at the concentration of one insect equivalent, females escaped more quickly than did the males. This could suggest a higher sensitivity of the females to the alarm pheromone or the existence of a residual female sex pheromone that could interfere with the escape behavior of the males. If so, females would respond fast to the defensive/alarm components in female extracts, whereas males would "hesitate" before escaping because they perceive the sex pheromone. Both sexes respond equally to male extracts because there is no sex pheromone.

As observed in social or gregarious insects, a defensive compound may serve alarm or aggregation functions when components are excreted in greater or lesser quantities (Calam and Youdeowei, 1968; Levinson and Bar Ilan, 1971; Ishiwatari, 1974, 1976; Lockwood and Story, 1985, 1986; Schmuck, 1987; Farine, 1989; Blum, 1996). Thus, a similar function of the secretion in *T. petiveriana* could be hypothesized. Unfortunately, because of the lack of insects to be tested, the activity of the lower concentration of the secretion remains unknown.

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IDENTIFICATION OF SEX PHEROMONE COMPOSITION OF CLICK BEETLE *Agriotes brevis* CANDEZE

MIKLÓS TÓTH,[1,*] LORENZO FURLAN,[2] VENYAMIN G. YATSYNIN,[3] ISTVÁN UJVÁRY,[4] ISTVÁN SZARUKÁN,[5] ZOLTÁN IMREI,[1] MITKO SUBCHEV,[6] TILL TOLASCH,[7] and WITTKO FRANCKE[7]

[1]*Plant Protection Institute, Hungarian Academy of Science*
Budapest, POB 102, H-1525, Hungary

[2]*Department of Agronomy, Entomology*
University of Padova
Agripolis, Via Romea 16, Legnaro PD, I-35020 Italy

[3]*Krasnodarskiy NIISKh im. P.P. Lukyanenko*
Krasnodar 12, Russia, 350012

[4]*Central Chemistry Institute, Hungarian Academy of Science*
Budapest, Pusztaszeri út 59/67, H-1025, Hungary

[5]*Agricultural University*
Debrecen, POB 58, H-4001, Hungary

[6]*Zoology Institute, Bulgarian Academy of Science*
Sofia, blvd Car Osvoboditel 1, BG-1000, Bulgaria

[7]*Institut für Organische Chemie der Universität Hamburg*
Martin-Luther-King-Platz 6, D-20146 Hamburg, Germany

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Abstract—Geranyl butyrate (GB) and (*E*,*E*)-farnesyl butyrate (FB) were identified in the pheromone gland extract of females of the click beetle, *Agriotes brevis* (Candeze) (Coleoptera: Elateridae) as the major sex pheromone components. Polyethylene vial dispensers containing 20–200 mg of a 1:1 mixture caught high numbers of beetles. Captures did not decrease even after 73 days of field exposure of dispensers. At sites where both *Agriotes sputator* L. and *A. brevis* were present, the above baits were selectively catching only *A. brevis*, despite the fact that GB is also the main pheromone component of *A. sputator*, suggesting that FB has a role in reproductive isolation. In the early part of the season, traps into which the insects could both crawl and fly captured more *A. brevis* than designs where the insects could only fly in. Trap design was not

* To whom correspondence should be addressed. E-mail: h2371tot@ella.hu or mtoth@mail.elender.hu

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important later in the season. This indicates the need for future development of a trap suitable for use throughout the whole season.

Key Words—Click beetles, *Agriotes brevis*, Coleoptera, Elateridae, *Agriotes sputator*, geranyl butyrate, (*E*,*E*)-farnesyl butyrate, pheromone, identification, field trapping, trap design.

INTRODUCTION

The click beetle *Agriotes brevis* (Candeze) (Coleoptera: Elateridae) is one of the economically most important click beetle pests in Europe. In fact, it can be considered the main pest on maize and other crops in Italy (Rusek, 1972a; Furlan, 1999; Furlan et al., 2000). In the Czech Republic and other European countries, it must also be considered a key species (Rusek, 1972b). According to sightings of adults, the presence of *A. brevis* is widespread in central and southern Europe (Platia, 1994).

The present study was undertaken to identify the chemical composition of the female-produced sex pheromone of *A. brevis*, in order to develop a species-specific pheromone trap for this species. Pheromone traps are used for the detection and monitoring of many insect pests, and in some cases the synthetic pheromone is used for direct control of populations through mating disruption. Pheromone trapping may be an easy, effective, and cheap method to determine precisely the distribution of this species and to implement IPM strategies (Furlan et al., 1997).

METHODS AND MATERIALS

Insects. Adult *A. brevis* specimens were collected from the colonies maintained at the San Donà laboratory according to methods described elsewhere (Furlan, 1998). Rearing in cages and vials started from females or larvae collected in Piemonte (northwestern Italy), Veneto (northeastern Italy), and Lazio (central Italy); the females obtained in overwintering cells were kept isolated in single vials with humid soil and some rye grass leaves until pheromone extraction was performed at the time when the male swarming was observed in the field.

Live insects were taken to the Budapest laboratory. Female sex pheromone gland extracts were prepared as described by Russian authors (Oleschenko et al., 1976; Ivaschenko and Adamenko, 1980), i.e., by carefully piercing the pheromone gland with a fine glass capillary and collecting the liquid inside into the capillary. The liquid samples obtained were dissolved in analytical grade hexane (MercK KGaA, Darmstadt, Germany) to make up stock solutions.

Gas Chromatography. Gas chromatography (GC) was performed on a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Palo Alto, California, USA) equipped with a flame ionization detector, using the following

columns: (1) SP 2340 fused silica (30 m × 0.32 mm ID; 0.20 μm; 1.5-m retention gap at the beginning of the column; Supelco Inc., Bellefonte, Pennsylvania, USA); split–splitless injector; temperature, 210°C; helium was used as the carrier gas at 1.5 ml/min; septum purge 2 ml/min; temperature program: 60°C, 1 min; 10°C/min to 100°C; 5°C/min to 220°C; held at this temperature for 20 min. (2) HP Ultra 1 cross-linked methyl silicone gum phase (25 m × 0.2 mm ID; 0.33 μm; 2.5 m retention gap at beginning of column; Hewlett-Packard); on column injector; carrier gas: helium at 1.5 ml/min; supply flow 30 ml/min; temperature program: 60°C for 1 min; 10°C/min to 100°C; 5°C/min to 220°C; held at this temperature for 40 min.

Coupled Gas Chromatography–Mass Spectroscopy. Coupled gas chromatography–mass spectrometry (GC-MS) was carried out by electron impact at 70 eV using a double focusing VG 70/250 SE mass spectrometer (Vacuum Generators, Manchester, UK) linked to a HP 5890 gas chromatograph. Separations were performed on a fused silica capillary column (50 m × 0.25 mm ID) coated with FFAP as the stationary phase (Macherey and Nagel, Düren, Germany). Helium was used as carrier gas. The column was operated at 60°C for 3 min and programed to 220°C at 3°C/min.

Traps. Bottle traps were funnel traps home-made from 2-liter transparent plastic bottles by cutting the bottle below the neck (ca. 10 cm) and placing the cut part upside down into the remaining bottom of the bottle (Figure 1). The resulting funnel entrance diameter was ca. 8 cm; the funnel hole diameter was ca. 2 cm. A transparent plastic sheet (10 × 10 cm) was placed in a vertical position immediately above the opening of the funnel. The bait dispenser was attached to the vertical plastic sheet so that the attractant-containing part was hanging into the large opening of the funnel.

VARb funnel traps were the plastic CSALOMON VAR funnel traps produced by the Plant Protection Institute, Budapest, Hungary, except that a transparent

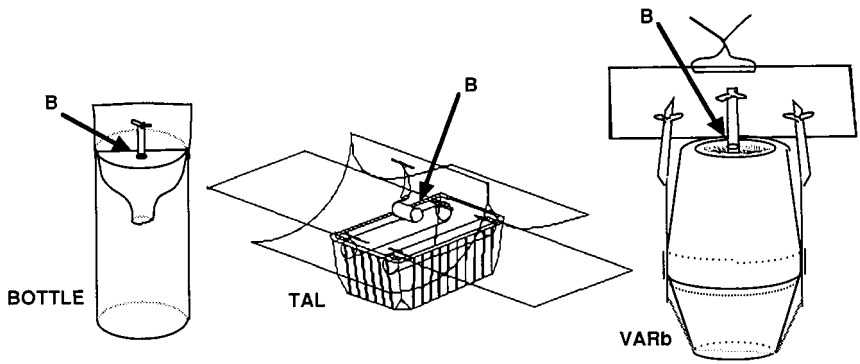


FIG. 1. Schematic diagrams of bottle, TAL, and VARb trap designs. B = position of bait dispenser.

plastic sheet (23 × 10 cm) was placed in a vertical position immediately above the opening of the funnel (Figure 1). A bait dispenser was attached to the vertical plastic sheet so that the attractant-containing part was hanging into the opening of the funnel. The funnel entrance was 13 cm ID, the hole of funnel was 3 cm ID and the height of funnel was 10 cm. Beetles falling down through the funnel were caught in a ca. 1-liter plastic container.

The TAL traps were modified pitfall traps. They consisted of a brick-shaped plastic box, a roof made of a folded plastic sheet into which the hanging bait was fixed and two other sheets on both sides of the plastic box that provided a smooth surface for beetles entering the trap and leading them into the plastic box. At the bottom of the box, a 10- × 16-cm sticky sheet was placed to make escape from the trap harder (Figure 1). The dimensions of the plastic box were: 17 cm long, 11 cm wide, and 6 cm high.

The YATLOR traps were made of plastic at the Italian laboratory and were similar in shape and size to the Estron trap described earlier (Oleschenko et al., 1987; Kudryavtsev et al., 1993).

Insects could get into the TAL and YATLOR traps by both crawling and flying, but could get into the VARb and bottle traps only by flying.

Chemicals. Geranyl butyrate (GB) was purchased from Bedoukian Inc. (Danbury, Connecticut, USA) and was >99% pure by GC. (*E*,*E*)-Farnesyl butyrate (FB) was synthetized. For the synthesis, (*E*,*E*)-farnesol (96%), butyryl chloride (98%), and (4-dimethylamino)pyridine (99%) were obtained from Sigma-Aldrich Kft. (Budapest, Hungary), while other chemicals were purchased from Reanal Finechemical Co. (Budapest, Hungary) and were of highest purity available. Analytical grade pyridine was dried over potassium hydroxide pellets. Column chromatography was carried out with 0.063- to 0.200-mm silica gel (Reanal Finechemical Co.). Proton NMR spectra were recorded on a Bruker WM-300 spectrometer at 300 MHz using tetramethylsilane as reference. For the synthesis of FB, butyryl chloride (9.9 ml, 95 mmol) was added to an ice-cooled solution of (*E*,*E*)-farnesol (15.2 g, 68 mmol), anhydrous pyridine (11 ml, 136 mmol), (4-dimethylamino)pyridine (0.20 g), and benzene (130 ml) while stirring. After stirring the mixture at room temperature overnight, it was diluted with hexane (150 ml) and poured onto ice-cold water (150 ml). The phases were separated and the aqueous layer extracted with hexane (50 ml). The organic layers were combined; washed with 15% HCl solution, water, and saturated $NaHCO_3$ solution; dried over Na_2SO_4; filtered; and concentrated. The crude product was purified by column chromatography using hexane as eluent to give 19.2 g (96%) of the title product as a pale yellow oil.

^{1}H NMR (CDCl3) 0.94 (t, $J = 7.3$ Hz, 3H), 1.59 (bs, 6H), 1.67 (s, 3H), 1.70 (s, 3H), 1.94–2.32 (m, 12H), 4.58 (d, $J = 7.1$ Hz, 2H), 5.08 (m, 2H), 5.34 (t, $J = 7.1$ Hz, 1H).

Bait Dispensers for Traps. Bait dispensers for the tests were prepared by adding the required amounts of synthetics into 0.7 ml polyethylene vials with lid (No. 730, Kartell Co., Italy) in hexane solutions. After having allowed the hexane to evaporate, the lid of the dispensers was closed and the dispensers were wrapped individually in pieces of aluminum foil. All dispensers were stored at -30°C until use.

Field Trapping. Field tests were conducted at several sites in Hungary, Italy, and Bulgaria. Traps were set up in blocks. Each block was comprised of one of each treatment. The distance between traps within a block was 5–10 m. The distance between blocks ranged from 50 to 100 m. Traps were moved one position forward within a block at each occasion when the traps were inspected; at the same time, captured beetles were recorded and removed.

Capture data were transformed to $(x + 0.5)^{1/2}$ and were analyzed by ANOVA. Treatment means were separated by Duncan's New Multiple-Range Test. In case one of the treatments was catching no beetles, the difference of the catch of other treatments from zero catch was analyzed by one-sample t test. All statistical procedures were conducted using the software packages StatView v4.01 and SuperANOVA v1.11 (Abacus Concepts, Inc., Berkeley, California, USA).

Description of Single Field Experiments. Preliminary tests were conducted from May 23 to August 8, 1997, in Eraclea, in the Veneto region of Italy in an alfalfa field by using bottle traps. Traps were inspected weekly over 77 days, providing 33 data points (11 inspections of 3 block replicates).

The ratio of GB and FB was evaluated in the following field tests: March 13–May 13, 1998, in Eraclea, Veneto region, Italy, by using TAL traps in an alfalfa field—traps were inspected every 6–7 days over 61 days, providing 9 data points (9 inspections of 1 block replicate); May 20–June 13, 1998, in Eraclea, Veneto region, Italy, by using bottle traps in a maize field—traps were inspected every 2–3 days over 24 days, providing 24 data points (7 inspections of 3 block replicate); March 28–June 8, 1998, in Debrecen, Hajdú-Bihar County, Hungary, by using VARb traps in an alfalfa field—traps were inspected every 8–9 days over 72 days, providing 32 data points (8 inspections of 4 block replicates); and April 2–May 15, 1998, in Eraclea, Veneto region, Italy, by using YATLOR traps in a sugar beet field—traps were inspected every 3–4 days over 43 days, providing 41 data points (13 inspections of 3 block replicates).

Catches with TAL versus Bottle Traps. Captures by traps baited with a 1:1 mixture of GB and FB (200 mg) were compared in the following experiments: March 30–April 26, 1998, in Eraclea, Veneto region, Italy, in a maize field—traps were inspected every 3–4 days over 27 days, providing 14 data points (7 inspections of 2 block replicates). April 26–July 7, 1999, in Eraclea, Veneto region, Italy, in an alfaalfa field—traps were inspected every 10–12 days over 72 days, providing 18 data points (6 inspections of 3 block replicates).

Other Field Tests. A 1:1 mixture of GB and FB was compared at different doses (20, 60, and 200 mg) from April 2 to May 19, 1999, in Eraclea, Veneto region, Italy, in a sugar beet field with YATLOR traps. Traps were inspected every 4–5 days over 45 days, providing 40 data points (10 inspections of 4 block replicates).

Bait longevity in the field was studied from February 29 to June 18, 2000, with YATLOR traps baited with a 1:1 mixture of GB and FB in a sugar beet field in Eraclea, Veneto region, Italy. Traps with fresh baits were added on April 5 and May 3. Aged baits were coming from traps operated at the same site preceding the test for the respective aging periods. Traps were inspected between April 5 and May 3 every 7–8 days over 28 days, providing 16 data points (4 inspections of 4 block replicates); and between May 3 and June 18 every 10–12 days over 46 days, providing 12 data points (3 inspections of 4 block replicates).

Monitoring at a site with both *A. brevis* and *A. sputator* populations was performed in Sofia, Bulgaria at the Botanic Garden of the Bulgarian Academy of Sciences from April 20 to July 27, 2000. Two TAL traps were used for *A. brevis* and two VARb traps for *A. sputator*. Traps were inspected each week. The *A. brevis* bait contained 30 mg of both GB and FB, while the *A. sputator* bait contained 30 mg of GB.

Seasonal monitoring for *A. brevis* was conducted in Eraclea, Veneto Region, Italy (March 4–June 30, 2000) in a sugar beet field with four YATLOR traps baited with 60 mg of an 1:1 mixture of GB and FB; baits were renewed at monthly intervals.

RESULTS AND DISCUSSION

Gas chromatographic analyses revealed two major peaks in the gland extract of female *A. brevis* in the relative ratios of 1:3, 1:2.8, and 1:2.4 for the northwestern, central, and northeastern populations, respectively (Figure 2). The retention times on both the SP-2340 and the Ultra-1 columns of the earlier eluting peak corresponded to the data of synthetic geranyl butyrate (GB), while the later eluting peak matched the retention times of (*E*,*E*)-farnesyl butyrate (FB). Mass spectra confirmed these assignments. The amount of the major pheromone component was estimated to be 5.5, 7.9, and 5.1 μg/female gland for the northwestern, central, and northeastern populations, respectively.

Apart from the above two compounds, GC-MS analyses revealed the presence of traces of geranyl hexanoate, farnesyl acetate, geranyl pentanoate, and geranyl 3-methylbutyrate in all gland extracts. Traces of β-farnesene could be detected only in extracts from the southwestern and northeastern populations.

Since the results did not show any substantial differences in the composition of the pheromone gland extracts between the three populations, it was decided to

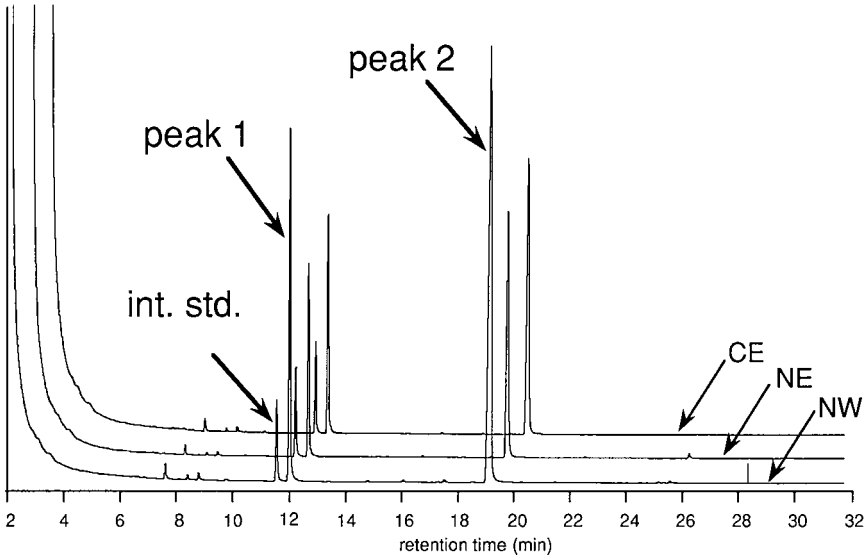


FIG. 2. GC trace of pheromone gland extracts of *A. brevis* populations on a SP-2340 capillary column. Internal standard was dodecyl acetate (50 ng). CE = central; NE = northeastern; NW = northwestern populations. Traces from different extracts are staggered by 0.3 min.

continue with testing the field activity of the two major compounds in the Veneto region of Italy.

In earlier field tests while screening known pheromone components of click beetle species in Italy, low catches of *A. brevis* males were occasionally observed in traps baited with GB (Furlan and Tóth, unpublished data), indicating that this compound might play a role in the pheromonal communication of the species, but was not sufficiently active for practical use.

Preliminary field tests set up in 1997 based on the first results of GC gland extract analyses showed that traps baited with a 1:1 mixture of GB and FB (5 mg each) caught a number of beetles (17.7 ± 7.8, mean $\pm$ SE), while those baited with FB alone (5 mg) caught only 1.5 ± 0.7 males. Traps with GB (10 mg; the dosage catching regularly low numbers in the previous screening tests) alone caught 0.1 ± 0.1 male beetles.

In tests comparing the field activity of the two compounds and their mixtures (Table 1), in Italy the 1:1 and 10:1 mixtures of GB and FB tended to show significantly higher catches of *A. brevis* than the 1:10 blend. With one exception, the single compounds caught the lowest numbers. In the Eraclea test in 1998, the activity of the 10:1 blend did not differ from that of the 1:1 blend and GB or FB alone. When used alone, there was a tendency of GB to attract more, but the

TABLE 1. CAPTURES OF *A. brevis* AND *A. sputator* BEETLES IN TRAPS BAITED WITH GERANYL BUTYRATE, (*E*,*E*)-FARNESYL BUTYRATE, AND THEIR MIXTURES IN FIELD TESTS[a]

Bait (relative amounts in bait: in 1998 100 = 5 mg; in 1999 100 = 10 mg)		Beetles captured (N, mean ± SE)			
		A. brevis			*A. sputator*
Geranyl butyrate	(*E*,*E*)-Farnesyl butyrate	Parc/Greggio 1998	Eraclea 1998	Eraclea 1999	Debrecen 1998
	100	1.3a ± 0.6	4.9*a ± 2.3	3.4a ± 0.6	0.2a ± 0.1
100		3.4ab ± 1.1	7.8ab ± 2.4	9.8b ± 1.7	10.9d ± 1.3
100	10	7.4cd ± 2.0	7.4ab ± 1.9	31.9d ± 4.6	1.9c ± 0.3
100	100	11.6d ± 2.7	13.2b ± 5.3	41.4d ± 5.6	1.3bc ± 0.2
10	100	6.1bc ± 1.4	5.3a ± 2.1	16.9c ± 2.2	0.7ab ± 0.2
		0.0* ± 0.0	0.0* ± 0.0	not tested	not tested

[a] Means with same letter within one column are not significantly different at $P = 5\%$ by ANOVA followed by DNMRT. Means with an asterisk within one column do not differ from zero catch at $P = 1\%$ by one-sample t test.

difference was significant only in one case. No other click beetle species were caught in the course of these tests in Italy. It was concluded that a 1:1 ratio of the two compounds is highly attractive towards *A. brevis*, and this mixture was used in the ensuing dosage tests.

To our surprise, in the tests in Hungary, no *A. brevis* were captured with the 1:1 blend. Instead, large numbers of the closely related *A. sputator* were caught in traps baited with only GB (Table 1). Other combinations caught negligible numbers, although catches in traps baited with mixtures containing large amounts of this compound caught slightly better than traps with little or no GB. This compound is the known main pheromone component of *A. sputator* (Kudryavtsev et al., 1993, Siirde et al., 1993; Yatsynin et al., 1986, 1996) and attracted large numbers of this species into traps also in other tests in Hungary (Tóth and Furlan, unpublished data).

The presence of FB in the ratios tested was strongly inhibitory towards *A. sputator*. This suggested a role for this compound in maintaining reproductive isolation between *A. brevis* and *A. sputator* in regions where they cooccur. In a test in 2000 aimed at monitoring the flight pattern of *Agriotes* spp. throughout the spring and summer seasons in Bulgaria, traps baited with the 1:1 blend caught a total of 364 *A. brevis* and no *A. sputator*, while traps baited with GB alone caught 2 *A. brevis* and 52 *A. sputator*, respectively. Although a direct comparison of catch figures is not possible since the two types of baits were set up in different trap designs in this test, this apparent specificity of the respective trap and bait combinations indirectly confirms the hypothesis on the role of FB in reproductive isolation between the two species.

TABLE 2. CATCHES OF *A. brevis* BEETLES IN TRAPS BAITED WITH FRESH OR FIELD-AGED DISPENSERS CONTAINING DIFFERENT DOSES OF 1:1 MIXTURE OF GERANYL BUTYRATE AND (*E*,*E*)-FARNESYL BUTYRATE[a]

Aged in field (days)	Amount in bait (mg)	Beetles caught (*N*, mean ± SE)	
		April 5–May 3	May 3–June 18
0 (fresh)	30	68.75a ± 6.78	11.08a ± 3.04
	60	89.50a ± 11.99	7.92a ± 2.81
31	30	not tested	7.42a ± 2.48
	60	not tested	7.83a ± 2.31
42	30	65.44a ± 7.97	not tested
	60	77.19a ± 8.39	not tested
73	30	not tested	9.50a ± 3.27
	60	not tested	8.91a ± 4.30

[a] For significance see Table 1.

In a 1999 test comparing different doses of the 1:1 mixture of GB and FB, traps with 20-mg, 60-mg, and 200-mg doses caught 19.5 ± 2.6, 21.1 ± 2.5, and 23.7 ± 3.8 beetles (mean ± SE), respectively. No significant difference was found between the means (ANOVA, DNMRT, $P = 5\%$). Unbaited traps captured 1.1 ± 0.3 insects, significantly lower than catches with all dosages of the baited traps.

In longevity tests of dispensers, no difference was observed at any of the two dose levels tested between the performance of fresh or aged baits even when aged for >73 days (Table 2).

In conclusion, a 1:1 mixture of GB and FB in 30- to 60-mg amounts in polythene dispensers can be recommended as a bait for *A. brevis* traps for agricultural use. In preliminary tests conducted throughout several months during the spring and summer, the presence of adult insects was shown from the beginning of March to the end of May (Figure 3) with a peak in the second half of April. The bait composition described in the present study clearly shows good perspectives for agricultural usage.

In the course of these studies, several trap designs were used: in some designs insects could get in without flying ("crawl-in" traps), in some others only by flying ("fly-in" traps). In a preliminary direct comparison of crawl-in and fly-in trapping devices, it was found that during the first half of the flight period many more *A. brevis* were captured in the crawl-in traps, while later in the flight the fly-in traps worked equally well (Table 3). This phenomenon might have resulted from the possibility that earlier in the season with cooler temperatures, *A. brevis* adults could not take wing so easily as later with warmer temperatures, although no direct observation of insect behavior was conducted in this study. In any case, more detailed studies, carried out at several sites in the geographical range of the species, are needed to study the influence of trap designs before an *A. brevis* trap

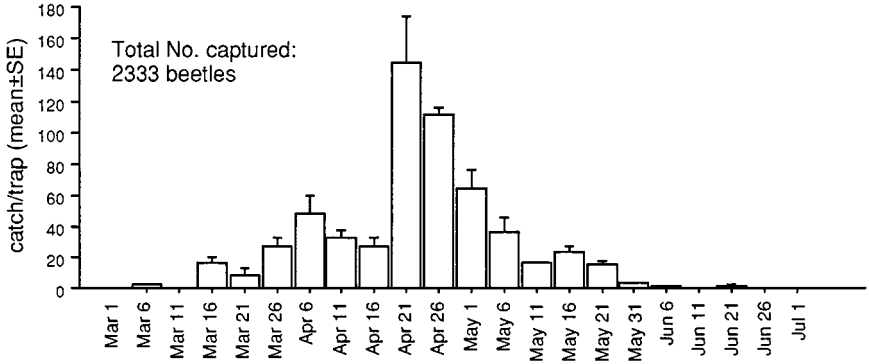


FIG. 3. Seasonal captures of *A. brevis* in traps baited with a 1:1 mixture of geranyl and (*E*,*E*)-farnesyl butyrate in Italy.

for general use can be fully developed. For a sensitive monitoring device, both the bait and the trap design should be optimized for maximal effect in catching the target pest.

Of the two compounds now identified as pheromone components of *A. brevis*, GB has already been described as a pheromone component in several *Agriotes* spp., i.e., *A. gurgistanus* Fald. (Yatsynin et al., 1980, 1996), *A. lineatus* L. (Yatsynin et al., 1991), and *A. ponticus* Step. (Yatsynin and Rubanova, 1990). The presence of this compound has also been shown in pheromone gland extracts (without data on behavioral activity) of *A. medvedevi* Dolin and *A. obscurus* L. (Yatsynin et al., 1996). In *A. sputator*, GB is clearly the main behaviorally active pheromone component (Yatsynin et al., 1986; Kudryavtsev et al., 1993; Siirde et al., 1993), while the behavioral significance of traces of FB found in gland extracts is not clear (Yatsynin et al., 1996). Closely resembling the results of pheromone analysis of *A. brevis* in this study, GB and FB together compose the pheromone of *A. meticulosus* Candeze (Yatsynin et al., 1991, 1996). Both GB and FB were also detected

TABLE 3. CATCHES OF *A. brevis* BEETLES IN TRAPS OF TWO DIFFERENT DESIGNS BAITED WITH 10 mg OF 1:1 MIXTURE OF GERANYL BUTYRATE AND (*E*,*E*)-FARNESYL BUTYRATE[a]

	Beetles caught (N, mean ± SE)	
Trap design type	March 30–April 26, 1998	April 26–June 28, 1998
TAL	16.5a ± 3.9	2.9a ± 0.9
Bottle	0.4b ± 0.2	3.3a ± 1.1
P (t test)	<0.001	0.3779

[a] Significance: Student's t test.

as minor components in gland extracts of *A. proximus* Schwarz and *A. ustulatus* Schaller (Yatsynin et al., 1996), where their behavioral relevance remains to be clarified. The presence of only FB also has been shown in gland extracts of *A. infuscatus* Desbr. (Yastsynin et al., 1996).

Consequently, the pheromone composition of *A. brevis* described in the present study seems to be well integrated into the general scheme of biologically active terpene esters of the genus *Agriotes* (see also Borg-Karlson et al., 1988): The biogenetic principle in the formation of specific pheromone components is based on the combination (esterification) of acyclic isoprenoid alcohols with short chain acids. Disposable variants are provided in the number of isoprene subunits and double-bond configurations at the terpene site (Tolasch and Tóth, unpublished data) and on chain length and methyl branching at the acid moieties.

In conclusion, we demonstrated the usefulness of the mixture of GB and FB formulated in polythene vial dispensers for the development of an *A. brevis* pheromone trap usable in agricultural practice. The issue of the optimal trap design still needs to be addressed, as in the present tests a crawl-in design was better in the early part of the flight period, while both crawl-in and fly-in designs performed well later on. The use of a crawl-in design later in the season has the drawback that occasionally large numbers of predatory beetles (adults and larvae of carabids, silphids, etc.) may also be captured, probably due to the volatiles from carcasses of insects captured earlier in the traps. Efforts for integrating design characteristics to develop an optimal trap design for click beetles are under way.

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PHEROMONE-BASED TRAPPING OF WEST INDIAN SUGARCANE WEEVIL IN A SUGARCANE PLANTATION

ALLAN C. OEHLSCHLAGER,[1,*] LILLIANA GONZALEZ,[1] MANUEL GOMEZ,[2] CARLOS RODRIGUEZ,[1] and ROMANO ANDRADE[1]

[1]*ChemTica Internacional, S. A.*
Apdo. 159-2150, San Jose, Costa Rica
[2]*Hacienda Juan Viñas, Costa Rica*

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Abstract—Attraction of *Metamasius hemipterus* (Oliver) to gallon and bamboo traps baited with insecticide-treated sugarcane, the male-produced pheromone, 4-methyl-5-nonanol, and 2-methyl-4-heptanol is more efficient if ethyl acetate is added. The optimal traps are ground-level gallon traps baited with insecticide-laced sugarcane, pheromone, and ethyl acetate. Capture rates of ground-level gallon traps are doubled by placing an insecticide-laced pad under the trap, but significantly decreased by placing the trap on a stick above ground. The efficiency of ground-level gallon traps is the same as ground level ramp traps. Mass-trapping *M. hemipterus* in newly planted sugarcane using ground level bamboo traps baited with insecticide-laced sugarcane and pheromone over six months revealed populations were low for the first two months, became maximum at five months, and declined thereafter. Capture rates of traps bordering newly planted and mature sugarcane were not significantly different from capture rates of traps in the interior of the plots. Capture rates of bamboo traps containing only insecticide-laced sugarcane and deployed at 30 traps/ha averaged 6 weevils/trap/week compared with 66 weevils/trap/week for traps additionally containing pheromone lures and deployed at 5 traps/ha. Capture rates for bamboo traps baited with insecticide-laced sugarcane and pheromone and deployed at 10 and 15 traps/ha were 43 and 38 weevils/trap/week, respectively. Total captures were higher in those plots with a higher density of insecticide-laden sugarcane and pheromone baited traps, and the differences were approximately proportional to trap density in the range of 5–15 traps/ha. Capture rates of traps containing insecticide-laced sugarcane and pheromone were always higher than of traps containing only insecticide-laced sugarcane, but in the first two months after planting the differences were much greater than in months 3–6 after planting.

* To whom correspondence should be addressed. E-mail: chemtica@racsa.co.cr

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Key Words—4-Methyl-5-nonanol, 2-methyl-4-heptanol, aggregation pheromone *Metamasius hemipterus*, West Indian sugarcane weevil, pheromone trapping, sugarcane.

INTRODUCTION

The West Indian sugarcane weevil, *Metamasius hemipterus sericeus* (Oliver), is a pest of sugarcane, *Saccharum officinarum* L., in regions of Central and South America above 1100 m in which climatic conditions provide cool and humid conditions for weevil reproduction and where crop cultivars may be restricted to varieties that are susceptible to weevil attack (Badilla et al., 1985). Weevils are attracted to the cut ends of sugarcane stalks (billets) laid in the ground during replanting. Females oviposit in and larvae mine the replanting stock, thus destroying nodal tissue and decreasing germination frequency. In some regions, damage to billets can reach as high as 60% and can result in production losses estimated at over 25% (Badilla et al., 1985). Development time from egg to emergent adult is approximately two months (Badilla et al., 1985).

Recommended practice to manage economically damaging populations of this weevil is to place bamboo traps containing insecticide-treated sugarcane in fields at planting for two months. In this work, we initially examined the relative efficiency of different trap designs, trap elevation, and baiting protocols. We also examined the relative efficiency of different mass-trapping protocols by using ground-level bamboo traps baited with insecticide-laced sugarcane or this component as well as male-produced aggregation pheromone (Perez et al., 1997).

METHODS AND MATERIALS

Field Site. Experiments were carried out in the 1200-ha Hacienda Juan Viñas sugarcane plantation in central Costa Rica. This is an elevated wet tropical region. The plantation has sugarcane of all ages, with most plantings bordered by roads. Mass-trapping plots were newly replanted sugarcane surrounded on three sides by 8-months-old sugarcane and on the fourth side by a road bordered by 8-month-old sugarcane. Mass-trapping plots were separated by at least 100 m of 8-month-old sugarcane.

Traps. Bamboo traps consisted of halved 4–5 cm diameter 0.8 m bamboo into which were cut on each side $\sim$0.6-m $\times$ 0.5-cm-wide slits for insect entry. Into these traps were placed four or five pieces of halved sugarcane stalk ($\sim$20 cm) previously immersed in Sevin 80 (3% A.I., 1-naphthyl *N*-methylcarbamate). Pheromone lures were laid in the trap with the sugarcane. Each trap was bound together with two pieces of wire, placed on the ground, and covered with litter from the

field to preserve moisture. Gallon traps were 4-liter yellow plastic containers with 10-cm × 15-cm windows cut in each large side ~5 cm from the bottom to allow insect entry. Ramp traps consisted of two yellow square molded plastic containers 13.5 × 13.5 × 3 cm mounted with open ends toward each other and separated by 5 cm. To the lower container were attached four ramps 12 × 12 cm that inclined between the top of the lower container and the ground. A picture of the trap is in Alpizar et al. (2000). Each trap contained four or five pieces of insecticide-treated halved sugarcane stalk (20 cm). Pheromone and ethyl acetate lures were hung from a wire just under the top of the trap.

Comparison of Gallon and Ramp Traps. Each trap contained five pieces of 20-cm-long halved sugarcane stalk previously immersed in Sevin 80 (3% AI), a pheromone lure, and an ethyl acetate lure. Traps were placed 50 m apart in 6- to 10-week planted sugarcane in Hacienda Juan Viñas, Costa Rica, November 30–December 14, 2001. Insects were counted and removed and traps were rerandomized between the first and second weeks.

Lures. Pheromone lures were commercially available Metalure [4-methyl-5-nonanol–2-methyl-4-heptanol (8:1)] formulated in membrane release devices that released ~3 mg/day. Ethyl acetate was formulated in membrane release devices that released ~100 mg/day (ChemTica Internacional, Apdo. 159-2150, San Jose, Costa Rica). Release rates were determined by weight loss under a daily temperature regime of 28 ± 2°C maximum and 17 ± 2°C minimum.

Statistics. Captures were analyzed for normal distribution and, where necessary, transformed to achieve homoscedasticity. ANOVA (fully factorial routine) was conducted using Systat 5.2.1. Means are always presented untransformed. Means followed by different letters in the figures are significantly different by Bonferonni t test ($P < 0.05$).

RESULTS AND DISCUSSION

A previous elucidation of the aggregation pheromone of *M. hemipterus* identified 4-methyl-5-nonano and 2-methyl-4-heptanol as the two components that yielded the highest biological responses in field trapping (Perez et al., 1997). While the participation of 4-methyl-5-nonanol in the aggregation pheromone of this weevil is undisputed, it was reported that either 2-methyl-4-octanol or 2-methyl-4-heptanol could be used with 4-methyl-5-nonanol to attract *M. hemipterus* in Colombia (Ramirez-Lucas et al., 1996). The present study used a mixture of 4-methyl-5-nonanol and 2-methyl-4-heptanol that has repeatedly been more attractive in Costa Rica than other mixtures of male-produced volatiles. Previous work established that addition of pheromone to insecticide-laced sugarcane-containing traps significantly increases capture rates of *M. hemipterus* (Giblin-Davis et al., 1996; Perez et al., 1997). Although other food additives have been investigated,

TABLE 1. SUMMARY OF EXPERIMENTS[a]

Exp.	Dates	Treatments	Design	Replicates
1	Oct 22–Nov 8, 1996	1 Bamboo traps (ground)—S, P 1 Gallon traps (ground)—S, P	CRBD	9
2	Nov. 8–22, 1996	1 Gallon traps, S, P 2 Gallon traps, S, P, EA	CRBD	10
3	Dec. 15–23, 1996	1 Gallon traps (~0.5 m above ground)—S, P, EA 2 Gallon traps (ground)—S, P, EA 3 Bamboo traps (ground)—S, P 4 Bamboo traps (ground)—S, P, EA	CRBD	10
4	Jan. 29–Feb 2, 1997	1 Gallon traps (ground)—S, P, EA (1.2 ml/day release) 2 Gallon traps (ground)—S, P, EA (1.2 ml/day release) with pad under trap	CRBD	7
5	Nov 30–Dec 14, 2001	1 Gallon traps (ground level)—S, P, EA 2 Ramp traps (ground level)—S, P, EA	CRBD	20
6 and 7	Sept. 1996 Feb 1997	30 bamboo traps (ground)/ha—S 15 bamboo traps (ground)/ha—S, P 10 bamboo traps (ground)/ha with—S, P 5 bamboo traps (ground)/ha with—S, P	CRBD	3 plots of 1 ha of each trap density

[a] Abbreviations: CRBD = complete random block design. S = 4–5 pieces of 20-cm-long halved sugarcane stalk previously immersed in 3% AI Sevin 80. P = pheromone lure emitting ~3 mg/day of 4-methyl-5-nonanol and 2-methyl-4-heptanol from a membrane lure containing these components in a ratio of 8:1. EA = ethyl acetate released at ~100 mg/day.

none has been found to be a better synergist for the aggregation pheromone than insecticide-laced split sugarcane stalk (Oehlshlager et al., unpublished results; Cerda et al., 1999).

Trap Optimization Experiments. Our initial experiment established that ground level gallon traps and bamboo traps were equally efficient when similarly baited and that insecticide-laced sugarcane in traps remained effective for at least two weeks (experiment 1; Figure 1). In this experiment, we found that captures during the second week were greater than captures during the first week. This is not unexpected since attractiveness of sugarcane to *M. hemipterus* is associated with its fermentation, the rate of which varies under field conditions. We next investigated the effect of addition of ethyl acetate to gallon traps (experiment 2; Figure 2). This experiment confirmed the earlier report that a combination of insecticide-laced sugarcane, pheromone, and ethyl acetate was more attractive than the binary combination of sugarcane and pheromone (Giblin-Davis et al., 1996).

Since it is common practice in sugarcane plantations to use ground-level bamboo traps to capture *M. hemipterus* (Badilla et al., 1985), we conducted a

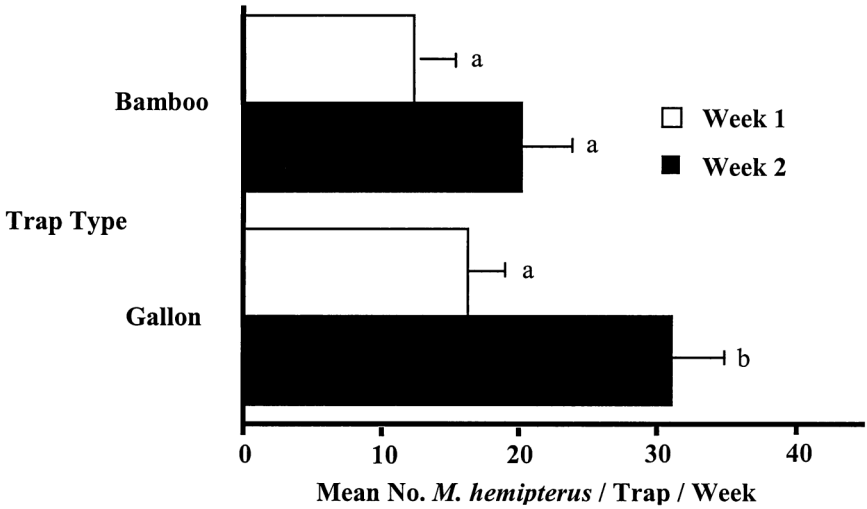


FIG. 1. Effect of trap type on *M. hemipterus* captures. Mean (+SEM) weekly capture of *M. hemipterus* in ground-level gallon and ground-level bamboo traps, each containing a pheromone lure and four pieces of 20-cm-long halved sugarcane stalk previously immersed in Sevin 80 (3% AI). Traps were placed 20 m apart in newly planted sugarcane (within one month of test) in Hacienda Juan Viñas, October 22–November 8, 1996. No significant difference between capture rates of the two trap types was detected in the first week (no statistical analysis presented), but in the second week ANOVA ($N = 9$) of log $(X + 1)$-transformed data gave $F_{1,16} = 4.49$, $P < 0.05$. Bars followed by different letters are statistically significant.

further experiment that determined that the addition of ethyl acetate to bamboo traps containing insecticide-laced sugarcane and pheromone increased capture rates (experiment 3; Figure 3). In experiment 3, we also determined that gallon traps baited with insecticide-laced sugarcane, pheromone, and ethyl acetate were more effective if placed on the ground than if elevated on sticks 0.5 m above the ground (Figure 3). The higher capture rate of ground-level traps is consistent with the preference of *M. hemipterus* for buried sugarcane stalk. The higher efficiency of ground-level traps is at variance with earlier reports in which it was observed that capture rates were higher in traps elevated above ground compared to those on the ground (Giblin-Davis et al., 1996; Cerda et al., 1999). The differences most probably reside in the ease with which *M. hemipterus* can enter the present ground-level traps. The present ground traps were surrounded by crop residue that was pushed along the sides of the gallon traps so that weevils landing on the ground near the trap could easily walk into the open sides of the trap. The higher capture rate of ground-level traps is consistent with our observations that *M. hemipterus* fly to the area of a trap, land, and then walk in. This is similar to

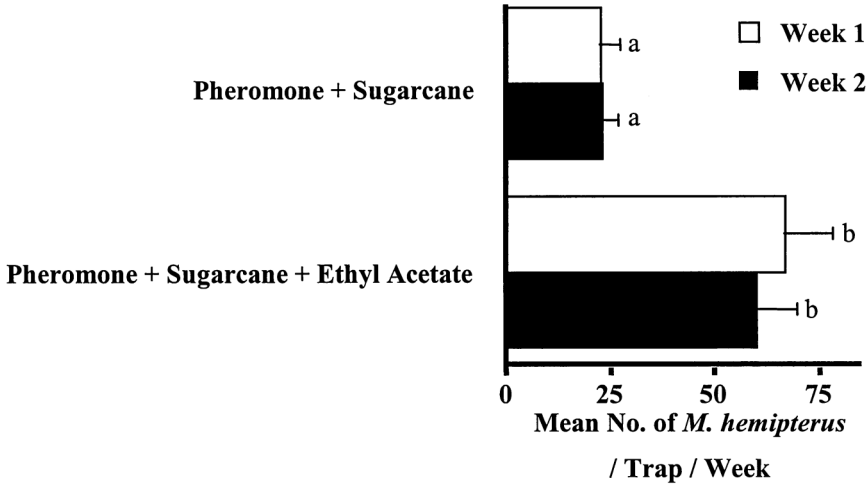


FIG. 2. Effect of semiochemical bait on *M. hemipterus* capture in gallon traps. Mean (+SEM) weekly capture of *M. hemipterus* in gallon traps each containing four pieces of 20-cm-long halved sugarcane stalk previously immersed in Sevin 80 (3% AI). Traps were placed 20 m apart in newly planted sugarcane in Hacienda Juan Viñas, November 8–22, 1996. ANOVA ($N = 10$) of week one captures gave $F_{1,18} = 7.88$, $P < 0.05$, week two $F_{1,18} = 7.15$, $P < 0.05$. Bars followed by different letters are statistically significant.

the behavior of *R. palmarum* that is also more efficiently captured if large landing surfaces are present within easy crawling distance of the traps (Oehlschlager et al., 1993a,b).

The above suggested that a ground-level killing agent might increase the efficiency of the trap. With the goal of intercepting weevils attracted to the area of the trap but not entering, we placed 20- × 20- × 0.5-cm insecticide-soaked sponge pads under gallon traps (experiment 4; Figure 4). Capture rates of ground-level gallon traps baited with insecticide-laced sugarcane, pheromone, and ethyl acetate were similar to equivalently baited gallon traps that were placed on insecticide-laced pads, and the insecticide-laced pads captured similar numbers of *M. hemipterus*. The trap–pad combination captured twice as many insects as the trap without pads. When this experiment was conducted in drier climates, it was found that if the pad is dry it does not retain significant numbers of insects compared to normal traps (D. Alpizar and M. Fallas, unpublished observations). In the present experiment, we used a very high (1.2 ml/day) release rate of ethyl acetate for all traps. It has been determined that capture rates in ground-level gallon traps baited with insecticide-laced sugarcane and pheromone and ethyl acetate steadily increase as the release rate of ethyl acetate is increased from 100 to 1.2 ml/day, the highest release rate studied (McDonald et al., 1996). The most effective trap is the insecticide-laced

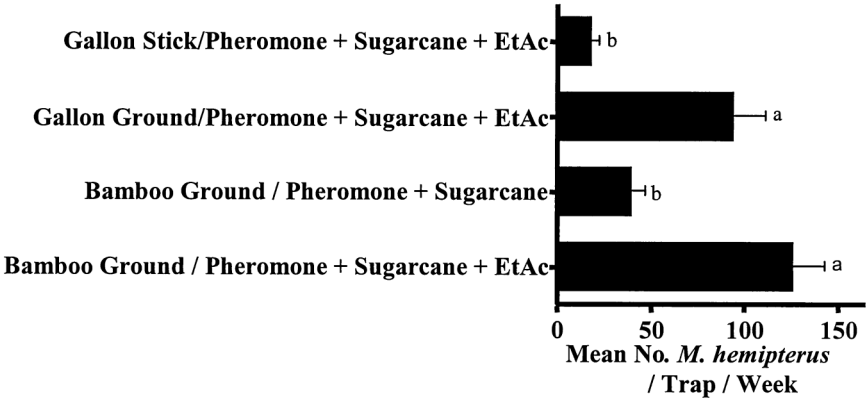


FIG. 3. Effect of trap type, trap elevation, and semiochemical bait on *M. hemipterus* captures. Mean (+SEM) weekly capture of *M. hemipterus* in bamboo or gallon traps in Hacienda Juan Viñas in 3-month-old sugarcane December 15–23, 1996. All traps contained four or five pieces of 20-cm-long halved sugarcane stalk previously immersed in Sevin 80 (3% AI). While all gallon traps additionally contained pheromone and ethyl acetate lures, bamboo traps were additionally baited with pheromone or pheromone and ethyl acetate lures. Additionally, gallon traps were filled to the lower window with Sevin 80 (3% AI) suspension that immersed the sugarcane. Gallon traps on sticks were ~0.5 m above ground level or on the ground. All bamboo traps were on the ground. All traps were ~20 m part between treatments and replicates. ANOVA ($N = 10$) gave $F_{3,32} = 15.38$, $P < 0.05$. Bars followed by different letters are statistically significant.

sugarcane–pheromone–ethyl acetate gallon trap placed on an insecticide-laced pad at ground level.

We have previously examined the relative effectiveness of the ground-level open gallon trap with a ramp trap, a plastic trap that allows weevils to crawl in easily and that is effective in the capture of *Cosmopolites sordidus* (Alpizar et al., 2000). These traps were equally effective when baited with insecticide-laced sugarcane, pheromone, and ethyl acetate. During the two-week trial (experiment 5), the total mean capture of *M. hemipterus*, in gallon traps and ramp traps was forty nine and thirty five, respectively [ANOVA ($N = 20$) $F_{1,18} = 1.82$, NS].

Mass-Trapping Experiments. The usual practice to manage populations of *M. hemipterus* in freshly planted sugarcane is to place 30 insecticide-laced sugarcane-containing bamboo traps/ha at the time of planting and leave them in place for two months. Since the above experiments demonstrated that bamboo traps containing insecticide-laced sugarcane and phermone were more effective, we conducted mass-trapping experiments using bamboo traps baited with both insecticiede-laced sugarcane and pheromone. Although addition of ethyl acetate lures to these traps would have made them even more effective, this component

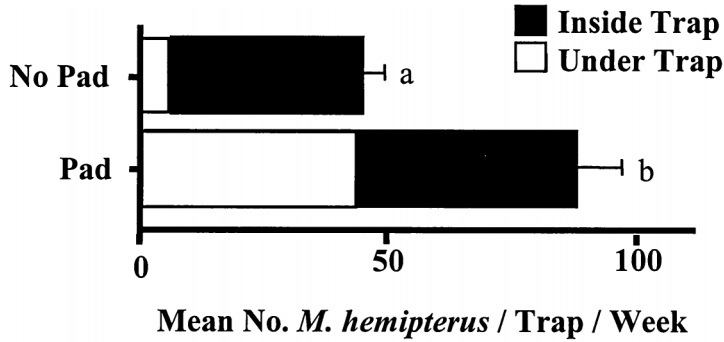


FIG. 4. Effect of the presence of an insecticide-laced pad under semiochemical-baited gallon trap on *M. hemipterus* captures. Mean (+SEM) weekly capture of *M. hemipterus* in gallon traps in Hacienda Juan Viñas sugarcane plantation, January 29–February 2, 1997. All gallon traps contained pheromone and 12 ethyl acetate lures (releasing ~1.2 ml/day of ethyl acetate) as well as four or five pieces of 20-cm-long halved sugarcane stalk previously immersed in Sevin 80 (3% AI). Traps were placed on ground with or without a 20- × 20- × 0.5-cm sponge pad presoaked in Sevin 80 (3% AI). ANOVA ($N = 7$) gave $F_{1,12} = 21.34$, $P < 0.05$. Bars followed by different letters are statistically significant.

was not used in the mass-trapping experiments because the release device available required frequent replacement, and it was not considered acceptable for extended field use. In the mass-trapping experiments, we deployed bamboo traps baited only with insecticide-laced sugarcane at a density of 30 traps/ha and bamboo traps baited with insecticide-laced sugarcane and pheromone at densities of 15, 10, and 5 and traps/ha (experiments 6 and 7, respectively). Each trap density was deployed in three 1-ha plots of newly planted sugarcane for 6 months.

The capture rate of *M. hemipterus* in all mass-trapping plots was low during first two months after planting but increased in months three to five before decreasing in the sixth month (Figure 5).

For two mass-trapping protocols, we examined the capture rates of bamboo traps baited with insecticide-laced sugarcane and pheromone that were located on the perimeter of the trapping plots with those traps that were located on the interior of the trapping plots. In the three 1-ha mass-trapping plots with 30 sugarcane-baited bamboo traps/ha, traps were placed on an approximately 18-m grid, with 16 traps on the perimeter of each plot and 14 traps located in interior positions. In each of the three mass-trapping plots with 15 insecticide-laced sugarcane- and pheromone-baited bamboo traps/ha, traps were placed on an approximately 25-m grid, with 14 traps in each plot on the perimeter and 9 traps located in interior positions. For these two trapping protocols, we found capture rates in perimeter traps to be within 20% of the capture rates of interior traps for each date during the entire six-month trial. That is, there was no evidence for migration of weevils

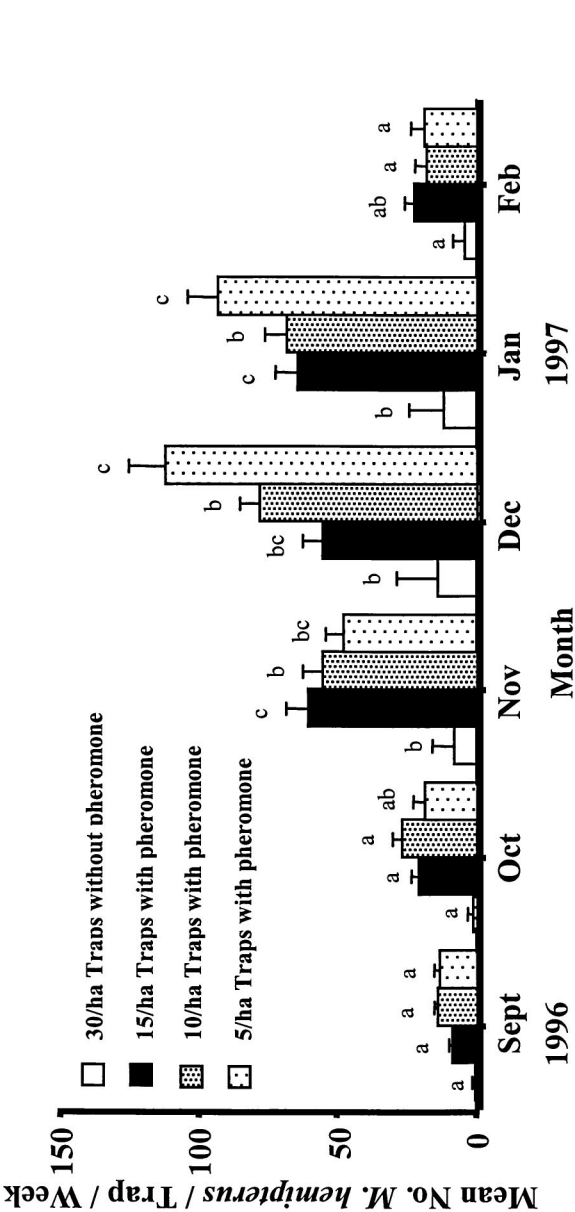


FIG. 5. Effect of trap bait and trap density on *M. hemipterus* captures. Mean (+SEM) weekly capture of *M. hemipterus* in ground level bamboo traps placed in 1-ha plots of newly planted sugarcane in Hacienda Juan Viñas, Costa Rica, September 1996–February 1997. All traps contained five pieces of 20-cm-long halved sugarcane stalk previously immersed in Sevin 80 (3% AI). Traps in plots with 15, 10, or 5 traps/ha additionally contained pheromone lures. Insects were counted and removed weekly, sugarcane was renewed biweekly, and pheromone lures were renewed when liquid was no longer visible in the lure. ANOVA for 30 traps/ha: $F_{5,834} = 24.93$, $P < 0.05$; for 15 traps/ha: $F_{5,414} = 20.64$, $P < 0.05$; for 10 traps/ha: $F_{5,274} = 17.99$, $P < 0.05$; for 5 traps/ha: $F_{5,129} = 30.73$, $P < 0.05$. Captures in plots containing the same trap density were averaged for each month and averages statistically compared month to month for the same trap density. Bars followed by different letters are statistically significant.

to the mass-trapping plots in the early stages of the trapping or for a build up of *M. hemipterus* in the center of the newly replanted mass-trapping plots during the latter part of the trapping. When similar trapping experiments were conducted in mature oil plam with *R. palmarum*, which is a much stronger flier, we found perimeter traps consistently captured two to three times more insects than those in the interior of the trapping area (Oehlschlager et al., 1995).

Bamboo traps baited only with insecticide-laced sugarcane and deployed at 30/ha were the least efficient, capturing an average of only 6 weevils/trap/week, while those baited with sugarcane and pheromone and deployed at 5/ha were the most efficient, capturing an average of 66 weevils/trap/week over the entire six-month trial. Traps baited with sugarcane and pheromone and deployed at 10 traps/ha (43 weevils/trap/week) and 15 traps/ha (38 weevils/trap/week) were of intermediate efficiency.

In the three 1-ha plots receiving 30 insecticide-laced sugarcane-containing bamboo traps (no pheromone lure)/ha, a total of 5217 weevils/ha were captured over the six-month trial. In the three 1-ha plots containing 15 insecticide-laced sugarcane- and pheromone-containing traps/ha, a total of 15,608 weevils/ha were captured during the six-month trial. This is ∼1.3 times more than the 11,636 weevils/ha captured in the plots containing 10 similarly baited traps/ha, and ∼2.3 times more than the 6676 weevils/ha captured in the plot containing only 5 similarly baited traps/ha. It is clear that use of insecticide-laced sugarcane- and pheromone-baited traps offers advantages and that use of as many traps as is economically feasible, at least up to 15/ha, results in removal of proportionally more weevils.

Month-by-month comparison of relative capture rates of bamboo traps baited only with insecticide-laced sugarcane with traps containing insecticide-laced sugarcane and pheromone revealed that in the first two months of the experiment, traps baited with insecticiede-laced sugarcane and pheromone captured ∼17 times as many insects as those with only insecticide-laced sugarcane (Figure 6). The relative efficiency of traps baited with insecticide-laced sugarcane and pheromone dropped to ∼6 times that of traps containing only insecticide-laced sugarcane during months 3–6 of the trials (Figure 6). This significant change is attributed to a difference in the attractiveness of the two components. In the early part of the trial, sugarcane had just been planted, and migrating weevils are expected to be primarily searching for mates and sites for oviposition. Additionally, in the early stages of infestation, there are few pheromone sources in the mass-trapping plots to compete with pheromone emanating from traps. During this period, it is reasonable that traps containing pheromone would be relatively more attractive than those without it. As colonization proceeds, location of unoccupied oviposition sites is likely to be dominant factor, and reliance on pheromone might be expected to decrease. Additionally, in the latter stages of infestation, there are many pheromone sources in the mass-trapping plots to compete with

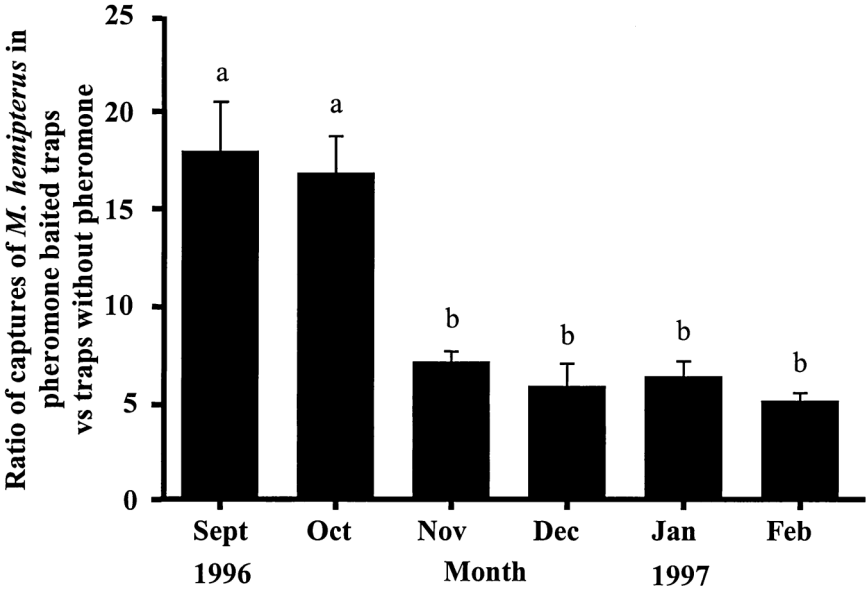


FIG. 6. Ratios of weekly capture of *M. hemipterus* in sugarcane vs. sugarcane and pheromone-baited bamboo traps over time. Ratios of weekly captures of *M. hemipterus* in 30 sugarcane-only bamboo traps containing only insecticide-laced sugarcane to weekly captures in bamboo traps containing insecticide-laced sugarcane and pheromone lures (experimental details as for Figure 5) placed at densities of 15, 10, and 5 traps/ha in newly planted sugarcane at Hacienda Juan Viñas, Costa Rica 1996–1997. ANOVA ($N = 3$) gave $F_{5,12} = 17.52$, $P < 0.05$. Bars followed by different letters are statistically significant.

pheromone emanating from traps. In these circumstances, one would expect differences in attraction between pheromone-containing and pheromone-free traps to decrease.

Acknowledgments—We wish to thank the technical staff at Hacienda Juan Viñas for excellent field assistance.

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INTERMALE VARIATION IN AGGREGATION PHEROMONE RELEASE IN *Prostephanus truncatus*

R. J. HODGES,* L. A. BIRKINSHAW, D. I. FARMAN, and D. R. HALL

Natural Resources Institute
University of Greenwich
Chatham Maritime
Kent ME4 4TB, United Kingdom

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Abstract—Intermale variation in pheromone signaling has been confirmed and quantified by measurements of pheromone produced by single adult male *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae). Males varied in both the amounts of the two components of their aggregation pheromone and the ratio of one component to the other. The mean rates of production of the pheromone components T1 and T2 were 1.9 and 0.5 μg/day, respectively. There were repeatable differences among males in the amounts of T2 produced and the proportion of T1 in the pheromone blend over two weeks. Of the 15 males studied, one released a large burst of pheromone in a short period, while the remainder, if they did release, did so over an extended period. This suggested that there may be two alternative release strategies and the significance of this is discussed.

Key Words—Aggregation pheromone, individual variation, sexual selection, larger grain borer, *Prostephanus truncatus*.

INTRODUCTION

The larger grain borer, *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae), is an important pest of farm-stored maize and dried cassava in Africa and Central America (Hodges, 1986; Markham et al., 1991). Once a male has reached a suitable food source, an aggregation pheromone is released consisting of two components, 1-methylethyl (*E*)-2-methyl-2-pentenoate and 1-methylethyl (*E*,*E*)-2,4-dimethyl-2,4-heptadienoate (Cork et al., 1991; Dendy et al., 1991), given the trivial names Trunc-call 1 and Trunc-call 2 (T1 and T2), respectively. Natural and synthetic pheromone are attractive to both females and males in the laboratory

* To whom correspondence should be addressed. E-mail: R.J.Hodges@gre.ac.uk

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(Boughton and Fadamiro, 1996; Hodges and Dobson, 1998) and field (Leos-Martinez et al., 1995; Scholz et al., 1997; Hodges et al., 1998). Synthetic pheromone is used as a lure in traps to monitor the pest (Richter and Biliwa, 1991; Fandohan et al., 1992; Pike et al., 1992) and also *Teretrius nigrescens* (Lewis) (Coleoptera: Histeridae), a predator of *P. truncatus* (Rees et al., 1990; Boeye et al., 1992).

It has been proposed that male *P. truncatus* produce aggregation pheromone primarily to attract females as potential mates and that other males respond opportunistically by aggregating, representing a fitness cost to the signaler in terms of increased competition for mates and possibly for food (Hodges et al., 1999). This hypothesis is supported by the observations that aggregation pheromone is only produced by males (Cork et al., 1991), males shut down signaling with pheromone after adult females arrive (Smith et al., 1996), and the sex ratio of beetles caught from the field is female-biased (Scholz et al., 1997; Hodges et al., 1998). Both laboratory and field bioassays have shown that *P. truncatus* can distinguish between the pheromone signals of different males and that some males are more attractive than others (Birkinshaw and Smith, 2000). The signals of individual males have never been chemically quantified, however. The temporal stability of signals is also unknown. Birkinshaw (1998) suggested that the relative attractiveness of a range of males was consistent over three days, but after five days, this order had changed (although insects were moved between jars of medium in between time).

In this study, we measured the signals from 3- to 5-day-old adults for 24 days in order to determine the degree of intermale variation in signaling and the stability of intermale differences over time, given fairly constant conditions.

METHODS AND MATERIALS

The beetles used were adults of a Ghanaian strain collected from the field in 1996 and cultured on yellow maize in a CTH room at $27 \pm 1^{\circ}C$, $60 \pm 5\%$ relative humidity on a 12-hr light–dark cycle. Newly emerged virgin adults, 1–2 days old, were removed from pupal cells. Males were selected according to the form of the clypeal tubercles (Shires and McCarthy, 1976) and placed on wheat flour for two days. Just before the start of pheromone collection, each male was placed on a single maize grain since males only produce pheromone in the presence of food (Cork et al., 1991; Smith et al., 1996). To provide the beetles with easy access to the maize, each grain was drilled to give a single, blind-ending tunnel (2 mm diam.).

Pheromone was collected from individual males under the same conditions of temperature and relative humidity. Beetles in maize grains or control grains without beetles were placed singly in glass vessels of 30 cm^3 capacity (Fisher Scientific) through which air was drawn at a rate of l000 cm^3/min by electrical diaphragm pumps (Capex Mk II; Charles Austin). The intake air was purified by passage

through a filter containing activated charcoal (20 × 2 cm, 6–18 mesh), and the output air was passed through filters containing Porapak Q (200 mg, 50–80 mesh; Phase Separations) to collect volatiles emitted. A glass round-bottomed flask (1000 cm^3) was also connected in the system, between the pump and collection filters, to act as a buffer against pressure variations induced by the pump. The air was pumped continuously during the test and the Porapak filters were changed at intervals of one, two, or three days over a period of 24 days (see Figure 1 below). Studies with two Porapak filters connected in series showed that both T1 and T2 were fully retained on the first filter for at least three days. Breakthrough of T1 was apparent after six days, but there was no breakthrough of T2 after 13 days.

Volatiles collected on the Porapak were eluted with dichloromethane (750 μl; Fisher Scientific Distol grade) and octyl acetate (5 μg) added as an internal standard. Samples were assayed by gas chromatography, using a fused silica capillary column (30 m × 0.32 mm ID) coated with CPWax 52CB (Carbowax equivalent; Chrompack), with helium carrier gas and flame ionization detection. The temperature was held at 60°C for 2 min, then programmed at 6°C/min to 230°C. The injector temperature was 200°C, and the detector temperature was 240°C. The sample (2 μl) was injected splitless and not concentrated to avoid loss of pheromone components. Data were captured and processed with EZCHrom V.6 software. Amounts of the two pheromone components were calculated by comparison of peak areas with that of the internal standard and application of the corresponding factors. The latter were derived by calibration of pure synthetic components against the internal standard at the 5-μl level. Peak identity was confirmed at intervals by gas chromatography–mass spectrometry (GC-MS; Finnigan MAT ITD700, Thermoquest) using similar chromatographic conditions. The threshold of minimum detectable quantities of pheromone was determined by visual estimation of the magnitude of the smallest reliably detected peaks.

In the first test, there were nine beetles and one control, and this test was then repeated. The results of the two tests were similar (i.e., did not form two distinct data sets, see Figures 2 and 3 below), and for statistical analysis they were combined, giving a total of 15 observations on male pheromone release, since three sets of data were missing due to mortality and a failure in sexing, and the two controls. The repeatability (r) of male differences in signal characteristics, both the absolute amounts of T1 and T2 and proportion of T1 in the blend, was calculated as r = variance between individuals/(variance between individuals + variance within individuals) (Du et al., 1987). The significance of the temporal stability of differences between the same male signal characteristics, absolute amounts of T1 and T2, and proportion of T1 in the blend were assessed by calculating the correlation coefficient between signals produced on day 7 and those produced by the same males on day 21. These days were chosen as they encompassed a period of steady pheromone output for most males.

RESULTS

During the first three days that the beetles were on maize, the pheromone collected on filters was below reliably quantifiable amounts and was estimated to be at or below 10 ng/beetle/day of either component (Figure 1). From day 5 onwards, detectable amounts of pheromone were released by most beetles (Figures 2 and 3) over extended periods, but in one case there was a very large burst of release. In this case, some 70 μg of T1 and 22 μg of T2 were released over a period of only two days, followed by little or no further production. The total amount released by this beetle during the period of study was greater than any other male (Figure 2 and 3), although similar to the 24-day cumulative values of several other males that released 50–60 μg of T1 and 15–20 μg of T2. No detectable amounts of pheromone were observed in the controls.

On average, almost four times as much T1 was produced as T2 (Table 1). The relative proportions of T1 and T2 were less variable than the absolute amounts of T1 and T2 among males. Furthermore, the differences that did exist in this blend among males were more repeatable than the differences in amounts of T1 or T2 (Table 1; Figure 4).

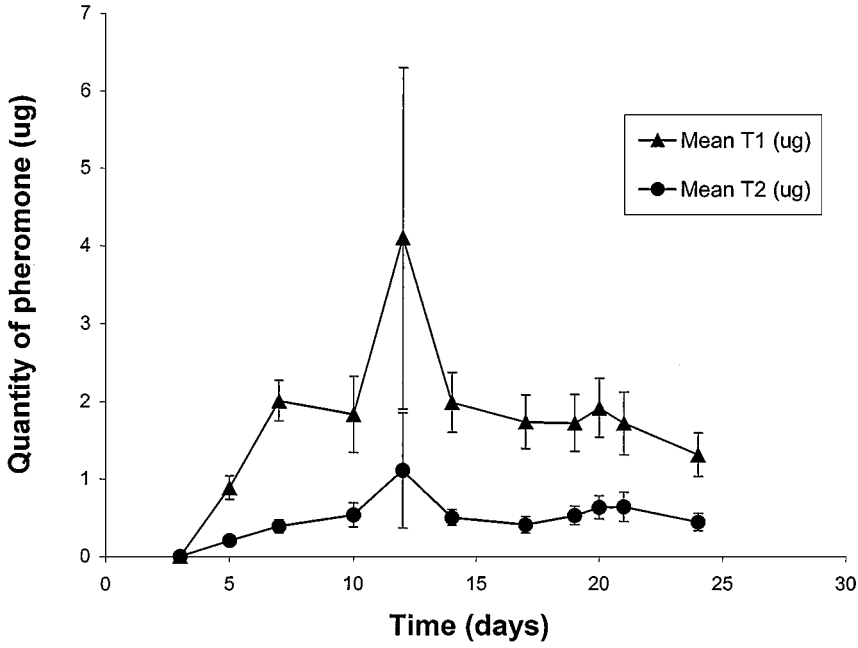


FIG. 1. Mean rate of pheromone output from male *P. truncatus* for pheromone components T1 and T2 (μg/day). Error bars are standard errors of the mean $N = 15$.

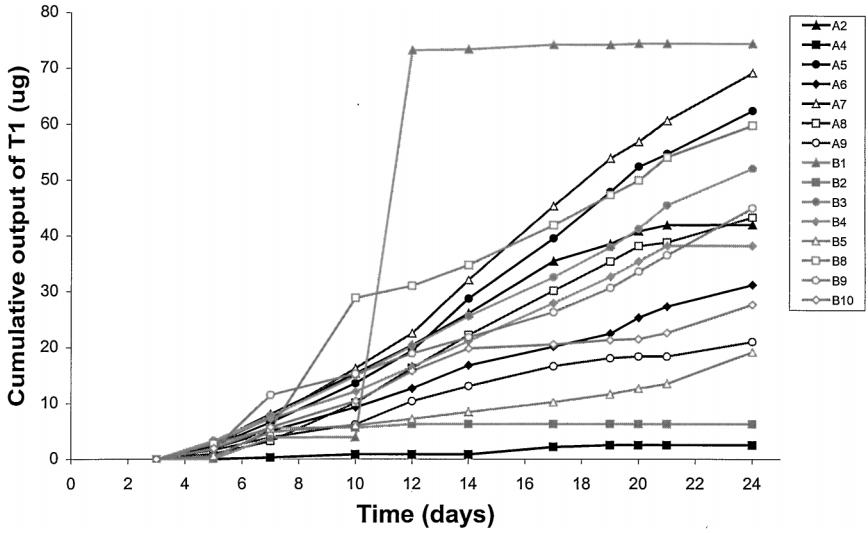


FIG. 2. Cumulative output of pheromone component T1 plotted individually for each male *P. truncatus* (A = first test, B = second test).

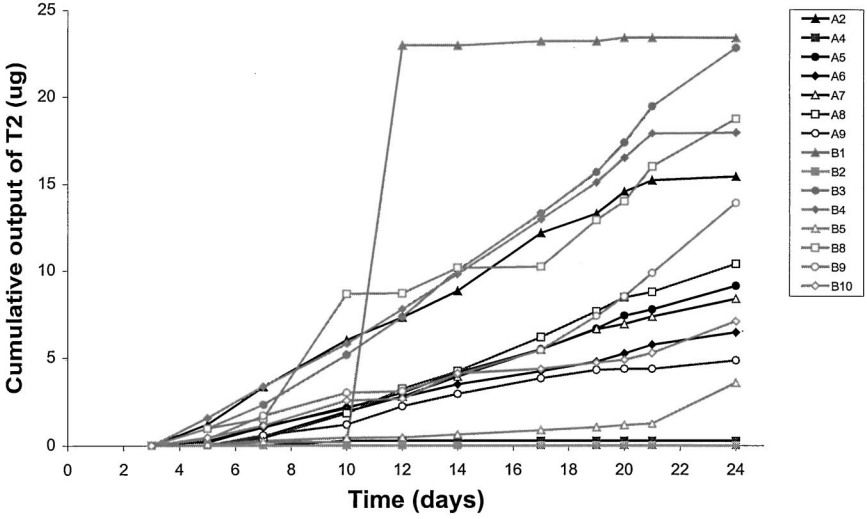


FIG. 3. Cumulative output of pheromone component T2 plotted individually for each male *P. truncatus* (A = first test, B = second test).

TABLE 1. SUMMARY STATISTICS OF QUANTITIES OF PHEROMONE COMPONENTS T1, T2, AND PERCENTAGE OF T1 IN AGGREGATION PHEROMONE BLEND PRODUCED BY INDIVIDUAL MALE *P. truncatus*

	T1	T2	T1[a]
Mean (of each male mean)[b]	1.9 (μg/day)	0.5 (μg/day)	81%
Coef. variation among males[b]	58%	75%	12%
Repeatability among males (r)[c]	0.44	0.49	0.57
Correlation coefficient between day 7 and day 21	0.32 ($N = 15$, NS)	0.57 ($N = 15$, $P < 0.01$)	0.69 ($N = 11$, $P < 0.005$)

[a] Only data where males produced a detectable signal were included.
[b] Using all data from day 5 onwards.
[c] Using data from day 7 to day 21.

No significant correlation ($r = 0.28$, $df = 14$, $P > 0.1$) was found between the rate of total pheromone production and the ratio of the two components among males (Figure 5).

DISCUSSION

During the first three days that male *P. truncatus* were placed on food, rates of pheromone production were below reliably quantifiable amounts in this study

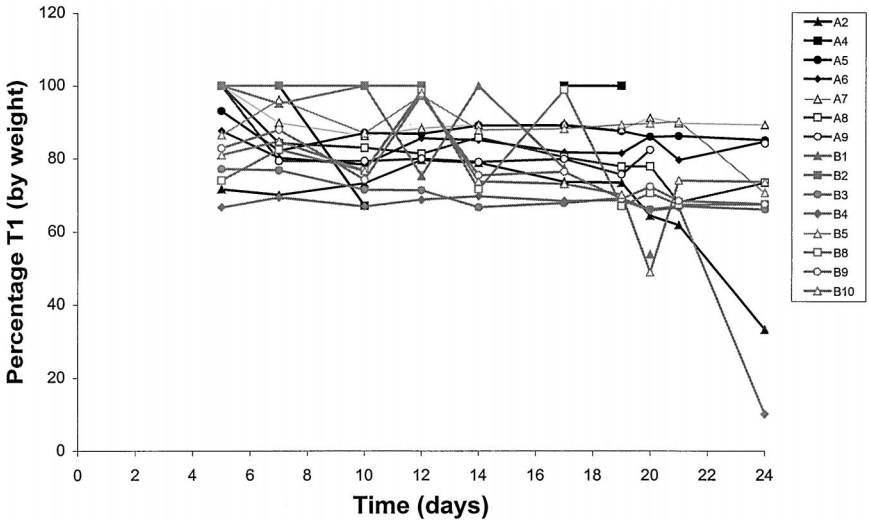


FIG. 4. Percentage of pheromone component T1 in the blend (by weight) plotted individually for each male *P. truncatus* against time.

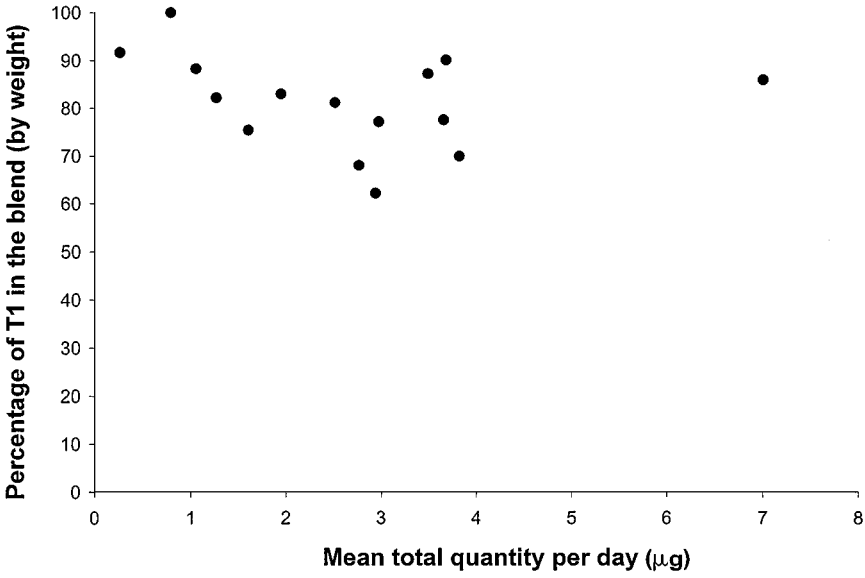


FIG. 5. Mean percentage pheromone component T1 in the blend vs. mean total quantity (T1 + T2) produced per day for each male *P. truncatus* for those days where measurable quantities of pheromone were produced.

($\leq$10 ng/beetle/day). However, bioassay studies have shown that both female and male *P. truncatus* respond to males that have only been present in grain for 24 hr (Hodges and Dobson, 1998); thus, it seems that the beetles respond to quantities of pheromone below our detection limits.

For most beetles, pheromone release extended over the 24 days of the study; however, one male released a single large burst of pheromone in this time. It is possible that this represents an alternative signaling strategy. The average weight of pheromone (T1 + T2) produce by males was 2.4 μg/day, and as the average weight of a beetle is about 3.5 mg (S. Addo, unpublished data), this amounts to 0.06% of body weight. However, in the extreme case, 92 μg was released in a two-day burst. This amounts to a daily rate equivalent to 1.3% of body weight. Little pheromone was produced after the large burst, suggesting that the high energy-costs involved could not be sustained, although this male did not release much more pheromone in two days than several others did in 24 days. If the pheromone burst is indeed an alternative strategy, then presumably the short attraction time is compensated for by the attraction of mates from greater distances. There may be more subtle advantages in avoidance of predation by the histerid beetle *T. nigrescens*, which shows a kairomonal response to *P. truncatus* pheromone (Rees et al., 1990; Boeye et al., 1992). However, this may only be clarified once host location by the predator is better understood.

Intermale variation in aggregation pheromone signaling, as suggested by the behavioral studies of Birkinshaw and Smith (2000), has now been confirmed directly, strengthening their hypothesis that features of the aggregation pheromone signal could be sexually selected. Compared to acoustic or visual signals, direct quantification of individual pheromone outputs are rare (but see Pope et al., 1984; Pavis and Barre, 1993; Miklas et al., 2000; Zhang et al., 2000). Recently, however, the pheromone release from a relative of *P. truncatus*, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), has been measured (Bashir et al., 2002). The male-produced aggregation pheromone of *R. dominica* also has two components, dominicalure 1 (D1) and dominicalure 2 (D2), and chemical structures are similar to those of T1 and T2 (Williams et al., 1981). Indeed, *P. truncatus* is attracted to traps baited with D1 and D2 (Hodges et al., 1983). Comparing the data sets, *P. truncatus* and *R. dominica* produce pheromone at comparable rates (typically 2–4 μg/day). The percentage of component 1 (D1 or T1) in the blend is lower from *R. dominica* than from *P. truncatus* if the components are compared by weight or in terms of the number of molecules of each. The absolute amounts of T1 and T2 are more variable than those of D1 and D2 reported by Bashir et al. (2002), although their ratios have about the same stability. The more variable values for *P. truncatus* were not attributable simply to the large burst of pheromone release in one of the males. Variation in pheromone outputs among male *R. dominica* has been shown to be connected to differences in the body weight (Bashir et al., 2002). Unfortunately, in the current study, the weight of the male *P. truncatus* was not recorded, although in previous work no link could be demonstrated between the weight of males and the attractiveness of their volatile pheromone signals (Birkinshaw, 1998).

The aggregation pheromone signals of male *R. dominica* are phenotypically plastic and can be altered by placing males on different plant hosts (peanuts compared to maize) and by crowding a male with many females (Bashir, 2000). It is already known that *P. truncatus* males greatly reduce their signal in the presence of females (Smith et al.,1996). The possible influences of plant host type on pheromone release by *P. truncatus* are as yet unknown, although males signaling on cassava were found to be less attractive than those signaling on maize in a walking bioassay (Birkinshaw, 1998).

The next logical step is to link chemical quantification of individual pheromone blends to bioassay data to find out which blends are the most attractive. This was recently attempted in *Rhyzopertha dominica* (Bashir, 2000). However, features of the blends, e.g., the ratio between the components, were not good determinants of responders' choices. However, in the case of *P. truncatus*, there is evidence that the blend is important. Artificial lures holding a total of 2 mg of T1 and T2, at a ratio of 2:1, released a blend that is a closer mimic of the natural pheromone than at a ratio of 1:1. Flight traps loaded with the 2:1 ratio lures captured significantly more beetles (Hodges et al., unpublished data).

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trans-BERGAMOTENES—MALE PHEROMONE OF THE ECTOPARASITOID *Melittobia digitata*

FERNANDO L. CÔNSOLI,[1,*] HOWARD J. WILLIAMS,[2] S. BRADLEIGH VINSON,[1] ROBERT W. MATTHEWS,[3] and MIRIAM F. COOPERBAND[1]

[1]*Department of Entomology*

[2]*Department of Chemistry*
Texas A&M University
College Station, Texas, USA

[3]*Department of Entomology*
University of Georgia
Athens, Georgia, USA

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Abstract—The first male-produced sex attractant pheromone in the parasitic Hymenoptera has been identified. The elaborate courtship behavior of *Melittobia digitata*, an idiobiont that parasitizes the last larval instar or the pupal stage of solitary bees and wasps, involves a series of coordinated movements of legs, wings, and antennae, initiated after the female is attracted to the blind, flightless male. We identified α- and β-*trans*-bergamotene as the active compounds of the male *M. digitata* sex attractant. Variation in the release of the sex pheromone by males and the pheromone load during aging is also described.

Key Words—Sex recognition, sex pheromone, sesquiterpene, parasitoid, courtship.

INTRODUCTION

Courtship behavior in the parasitic Hymenoptera has been much investigated, focusing especially on the evolution of displays and on reproductive strategies (Matthews, 1975; Assem and Jackmann, 1982; Assem, 1986; Ayasse et al., 2001). In only a few cases have cues eliciting these reproductive behaviors been identified (Quicke, 1997; Kainoh, 1999 for review). Among chalcidoids, courtship typically involves a reciprocal series of movements of the wings, legs, and antennae (Assem and Povel, 1973; Assem and Jackmann, 1982).

* To whom correspondence should be addressed. E-mail: f-consoli@tamu.edu

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Melittobia (Hymenoptera: Eulophidae) is one of these chalcidoids that has been thoroughly investigated (Evans and Matthews, 1976; Assem et al., 1982; Matthews et al., 1985; Gonzalez et al., 1996). All known species are polyphagous, gregarious ectoparasitoids, with a remarkable biology and sexual dimorphism (Dahms, 1984a,b). Females of the second type (short wing morph, SWM) never leave the confines of the host's nest. They will copulate and lay their eggs on the same host on which they developed. These eggs will develop into the type form (long wing morph, LWM or dispersive morph) that typically copulates before dispersing (Dahms, 1984b). Males of *Melittobia* are brown (females are black), blind, flightless, and the antennal scape is markedly differentiated (Dahms, 1984c). *Melittobia* males remain in the host cocoon and wait for the eclosion of their female siblings, with whom they copulate (Dahms, 1984b).

Courtship behavior of *Melittobia* is elaborate, involving a series of alternating movements of different appendages (wing fanning, leg wagging, and antennal rubbing/stroking). After mounting females, males typically fan their wings and wag mid- and/or hind legs while rubbing and clasping the female's antennae with their own antennae (Dahms, 1973; Evans and Matthews, 1976; Assem and Jackmann, 1982; Gonzalez et al., 1996; Guinan et al., 2000).

Unlike the male-oriented response to females as reported for many insects, courtship initiation in *Melittobia* begins with attraction of females to males. Females first contact males, and the response of virgin females to males has been suggested to be due to the presence of a male sex pheromone (Hermann et al., 1974; Gonzalez et al., 1985; Matthews et al., 1985). Interestingly, females also respond to males of different *Melittobia* species in laboratory tests (Matthews et al., 1985), suggesting that even relatively distantly related species might use the same or closely related pheromones.

Although the biosynthesis and endocrine regulation of pheromone production are well studied for some groups of insects, the physiology and chemistry of pheromones in parasitoids have received relatively little attention (Tillman et al., 1999). In the majority of the studies in which a chemical is indicated, the structure(s) is not identified, and in those cases where sex pheromones have been successfully isolated and identified, all have been female-produced (Quicke, 1997; Kainoh, 1999 for review).

Here we report on the identification of a male pheromone of *Melittobia digitata* that attracts females prior to initiation of courtship. We also provide data on the male's pheromone load and rate of release at different ages.

METHODS AND MATERIALS

Insects. Melittobia digitata was reared on blowfly pupae, *Neobellieria bullata* (Diptera, Sarcophagidae) (Carolina Biological Supply), under controlled conditions ($26 \pm 1^{\circ}C$; $60 \pm 10\%$ relative humidity; 14L:10D). Pupae of the host had

the anterior end of the puparium removed with the aid of a surgical blade before they were offered to females of *M. digitata* (one pupa, five females). After development, pupae of the parasitoid were collected and transferred to new vials for eclosion and mating, after which new hosts were offered.

Bioassays. We first tested the response of females of different ages to males. Virgin females from day 1 to 10 after eclosion were released individually into the bottom part of a 5-cm-diam. plastic dish, that was placed upside down on the top of a glass surface. Two circular openings on the lateral side of the dish were plugged with size #1-gelatin capsules (20 mm long $\times$ 6.6 mm diam.) (Electron Microscopy Sciences, Ft. Washington, Pennsylvania, USA). Each gelatin capsule had a small pinhole (1.5 mm diam.) on the end facing the interior of the dish. A single male was placed in one of the gelatin capsules while the other was used as a control. Attractiveness of males to females was measured by counting the number of females entering the male cell after 1 hr of their release. Females were released individually into each arena (25 arenas/age group), and each female was considered as a replicate.

Extraction of Males for Fractionation and Bioassays. Males of *M. digitata* were removed from the rearing containers during the pupal stage and individually transferred to gelatin capsules prior to emergence. After emergence, 0- to 1-day-old males were extracted in ethyl ether (1 μl ether/male) for 1 hr. Samples were collected, transferred to new vials, and stored at -80°C, if not immediately used.

The same testing arena described above was used to test the activity of the male extract, its fractions, and that of synthetic compounds. In these cases, a group of ten 1- to 2-day-old females was released in each plate. The test solution was applied to 3- $\times$ 3-mm squares of Whatman No. 1 filter paper, air dried, and inserted into the gelatin capsule. Ethyl ether was used as control, with 10–22 replicates for each treatment. For the experiments where the extract was tested against different fractions, each square was treated with 2 male equivalents. In experiments where synthetic compounds were compared to the whole male extract, squares were treated with standard volume (given by the area of the peaks in the sample), and the concentration used was later determined by using a known concentration of a synthetic compound as standard (59 ng/square).

Collection of Male Volatiles for Identification. Volatiles released by males of *M. digitata* were collected by using an all-glass collection system with Porapak Q 80/100 (Alltech Associates) as a trapping material. An in-line activated charcoal filter (16 cm $\times$ 4 mm) was used to purify the air before it entered the system. The filter was connected to a male joint by Tygon tubing, and the female joint was connected to the male joint of the collection chamber (100 ml). Both male and female joints (standard ground-glass joint, 14/20) of the collection chamber were plugged with glass wool to prevent insects from leaving this chamber. A second glass tube, containing a microcolumn (collection trap), was connected to

the female joint of the collection chamber. Microcolumns consisted of 6.5-cm-long glass tubes (0.15 mm ID) filled with 12 mg of Porapak Q. Both ends of the tubes were plugged with glass wool, and tubes were rinsed with methylene chloride and ethyl ether before each collection. For each collection, approximately 100 newly emerged males were placed in the collection chamber for 4 days. Air was passed into the system at 20 ml/min. The airflow was measured using a bubble meter, connected to the end of the microcolumn. Columns were washed with 100 μl ethyl ether, and samples were pooled and stored at -80°C before analysis.

GC Analysis and Separation. Samples were concentrated by collecting the desired fractions in an all-glass GC preparative system (Williams and Vinson, 1980) attached to a Varian 3700 GC, equipped with a 3% OV-101 Chrom 750 80–100, 4-m $\times$ 4-mm-ID column (185 to 260°C at 25°C/min). Fractions were collected at specific times after the injection of the sample and were divided as follows: fraction 1, compounds 1–3; fraction 2, compounds 4–8; fraction 3, compounds 9–11 (Figure 2).

Collection for Quantification. Males of known age (1, 2, 4, 6, 8, and 10 days old) were used for quantification of the volatiles released, using a solid-phase microextraction fiber (SPME) (Supelco, Bellefonte, Pennsylvania, USA). Individual variation in the release of the male pheromone of *M. digitata* was measured by isolating single males in a 250-μl glass LV insert (Alltech Associates, Deerfield, Illinois, USA), inside a 2-ml glass vial with Teflon-lined septum screw cap. The SPME was introduced into the insert, and the fiber exposed over 2 hr. After collection, the sample was immediately injected in splitless mode into a HP 6890 GC, equipped with a BP-20 carbowax column (25 m $\times$ 0.25 mm ID) (SGE, Ringwood, Australia) (initial temperature $=$ 50°C for 5 min; to 150°C at 15°C/min; to 185°C at 5°C/min; to 225°C at 20°C/min; 138 KPa He as the carrier gas). Five replications were taken for each age group.

We also assessed the pheromone load and release of groups of five males at different ages, because many single males would not release sufficient volatiles for the detection of both isomers of *t*-bergamotene. Males were placed in 250-μl glass inserts, and the SPME-collected volatiles were analyzed as described before. Three replicates were run for each age group. After collection of the volatiles with SPME, males were extracted in ethyl ether for quantification of the pheromone available at that particular stage (pheromone load). Ten microliters of ether was added to 5 males. After 30 min, the solvent was collected, and the males washed with a second 10 μl of ether. Samples were pooled, and 1 μl was injected into the GC (as above). The amount of α- and β-*trans*-bergamotenes collected with SPME or extracted with ether was calculated by using standards of known concentrations. The values obtained for release (SPME) were compared to those available in tissues of the insect (pheromone load—ether extract), and used to estimate the rate of pheromone release by this species.

GC and GC-MS. Analytical GC was done with Hewlett Packard 5890 or 6890 instruments with column injection and flame ionization detection. Columns used were SGE BP1 and BP20 (25 m × 0.25 mm ID), with helium as carrier gas at 138 KPa. Mass spectroscopy was performed on a Hewlett Packard GC-MS 5989B instrument using a SGE BP1 column (25 m × 0.25 mm ID), temperature programmed at 40°C for 9 min to 280°C at 15°C/min. NMR spectra were acquired on a Bruker ARX-500 instrument by using a 5-mm HCN or 3-mm HBB probe with $CDCl_3$ solvent.

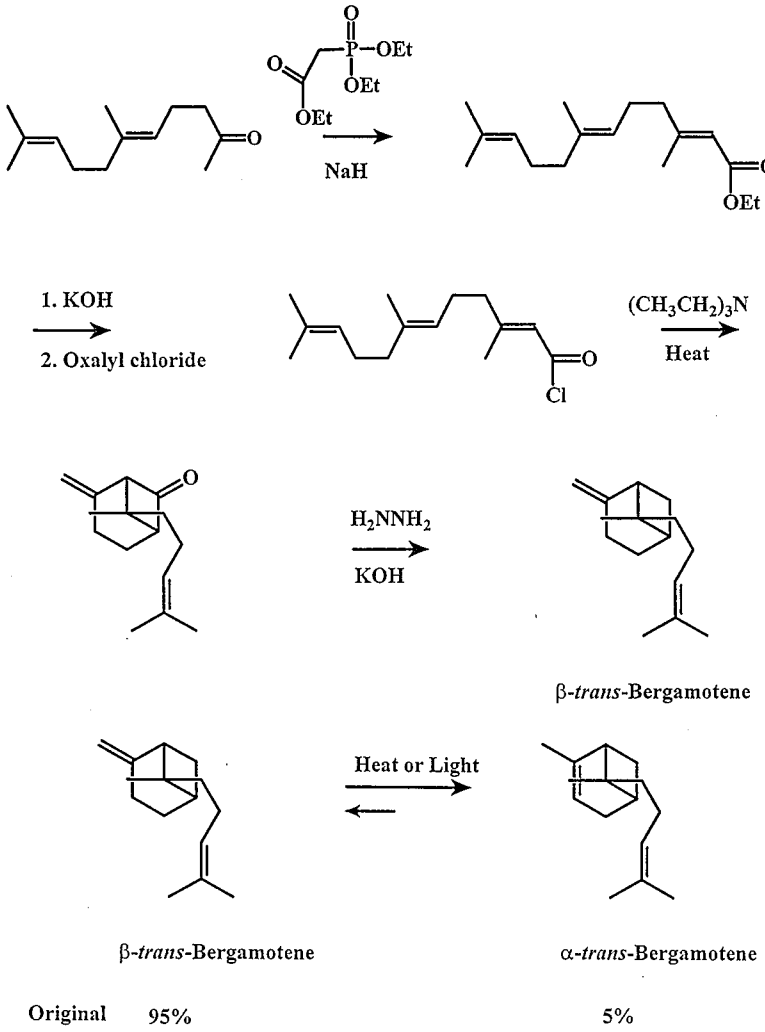
Isolation of α-trans-Bergamotene. *α-trans*-Bergamotene was initially obtained from essential oil of sweet basil, *Ocimum basilicum* (Aura Cacia, Weaverville, California, USA). The essential oil was distilled under vacuum (bp 100–130°C/9 mm Hg) and collected by preparative GC [Varian 3700, with a 3% OV-101 Chrom 750 column (80–100; 4 m × 4 mm ID); 185 to 260°C at 25°C/min].

Synthesis. *α-trans*- and *β-trans*-Bergamotene were prepared by the method of Kulkarni et al. (1987), which consists of reaction of the ylide of triethyl phosphonoacetate with geranyl acetone, followed by saponification, preparation of acid chloride using oxalyl chloride, cyclization by ketene formation with triethylamine in refluxing toluene, and ketone removal using hydrazine with potassium hydroxide (Scheme 1). As was the case for the insect-produced materials, it was found that *α-trans*-bergamotene was produced from the *β-trans*- form on standing (Scheme 1). Synthetic material was purified by the preparative GC described above.

RESULTS AND DISCUSSION

Bioassays. Females of *M. digitata* of different ages were equally attracted to males in our experimental arena (Figure 1), but 10–40% of females did not respond to males. This fact may be explained by the great variability found in the release of pheromone by individual males (see below).

The attractiveness of females to the whole-body extract of males was comparable to that of females that were offered males instead (Figures 1 and 3). We were able to detect 11 different volatiles released by males of *M. digitata* (Figure 2). Two fractions of the male pheromone were as attractive to females as the whole male extract (Figure 3). Purification of active components by preparative GC and analysis by GC-MS and NMR produced a definitive identification of *α-trans*-bergamotene (peak 1) and *β-trans*-bergamotene (peak 4). The spectral properties of synthetic materials were identical to those of the insect-released compounds and to literature values of Snider and Beal (1988) and Kulkarni et al. (1987) for the α and β forms respectively. *α-trans*-Bergamotene MS (m/z, %) = 204 (2.6), 119 (100.0), 93 (92.6), 69 (54.9), 91 (47.3), 107 (45.3), 105 (37.0), 55 (36.3), 79 (33.1), 120 (27.8); ^{1}H NMR (δ) 5.23 (br s, vinyl), 5.19 (br t, vinyl), 1.72, 1.68, 0.85 (CH_3); ^{13}C NMR (δ) 144.5 (s), 131.0 (s), 125.3 (d), 116.5 (d), 45.4 (d), 41.2 (s),



SCHEME 1. Synthesis of *β-trans*-bergamotene by the intramolecular cycloaddition reaction of vinylketenes with alkenes (Kulkarni et al., 1987), and transformation of *β-trans*-bergamotene to *α-trans*-bergamotene.

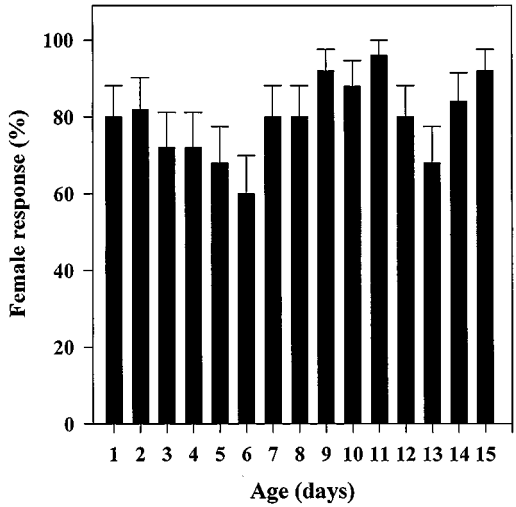


FIG. 1. Response (%) of *M. digitata* females at different ages to calling males.

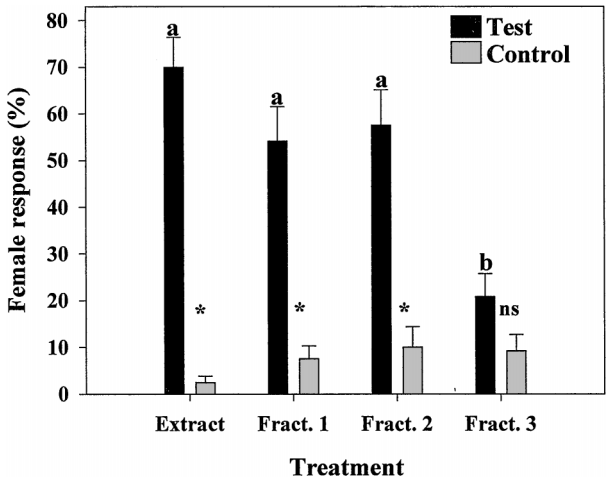


FIG. 2. Response (%) of *M. digitata* females to fractions of male extract [Bars x + SE] followed by different letters are statistically different by Tukey test at $P < 0.05$; ANOVA: $F = 10.03$; $df = 3, 47$; $P < 0.001$, $N = 12$. *Test is significantly different from control by the t test at $P < 0.05$ (fraction 1: compounds 1–3; fraction 2: compounds 4–8; fraction 3: compounds 9–11).

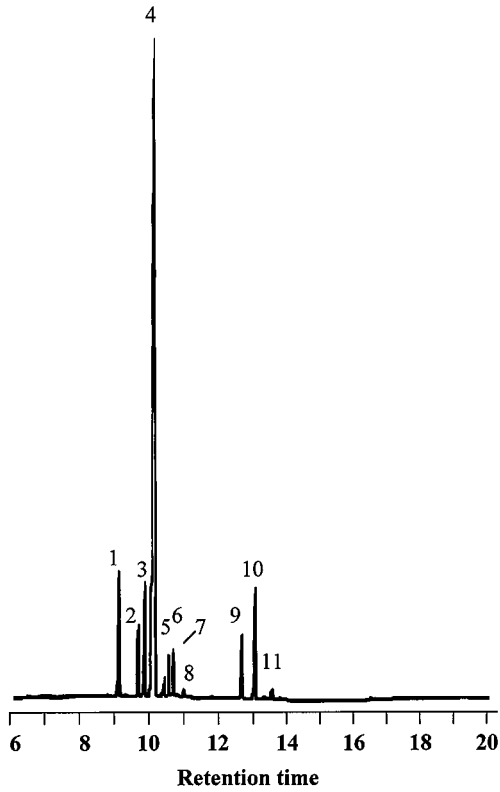


FIG. 3. Chromatogram (GC) of volatiles emitted by *M. digitata* males (peak 1 = α-*trans*-bergamotene, peak 4 = β-*trans*-bergamotene) (fraction 1: compounds 1–3; fraction 2: compounds 4–8; fraction 3: compounds 9–11) (column BP20, initial temperature 50°C for 1 min, to 155°C at 15°C/min, to 185°C at 5°C/min, to 225°C at 20°C/min).

39.0 (d), 38.6 (t), 31.6 (t), 31.0 (t), 25.7 (q), 23.8 (d), 23.0 (q), 17.6 (q), 17.5 (q). β-*trans*-Bergamotene MS (*m*/*z*, %) = 204 (3.0), 69 (100.0), 93 (58.9), 79 (31.6), 133 (30.0), 91 (29.8), 55 (25.8), 67 (25.7), 77 (21.9), 120 (21.8); ^{1}H NMR (δ) 5.18 (br t, vinyl), 4.65 (br s, vinyl), 4.58 (br s, vinyl), 1.72 (s, CH_3), 1.64 (s, CH_3), 0.73 (s, CH_3); ^{13}C NMR (δ) 152.2 (s), 131.1 (s), 125.1 (d), 106.0 (t), 50.2 (d), 43.8 (s), 38.7 (d), 38.2 (t), 31.7 (s), 27.1 (t), 25.6 (d), 23.8 (q), 23.5 (d), 18.6 (q), 17.6 (q). The retention times of synthetic bergamotenes and insect-related compounds were indistinguishable on a BP-1 column (35°C for 1 min, to 155°C at 15°C/min, to 185°C at 5°C/min, to 220°C at 20°C/min): synthetic α-*trans*-bergamotene and insect-related compound 1 (Figure 2) = 8.7 min; synthetic β-*trans*-bergamotene and insect-related compound 4 (Figure 2) = 9.1 min, and in between C_{14} (8.5 min) and C_{15} (9.4 min) standards; and on a BP-20 column (35°C for 1 min, to 155°C

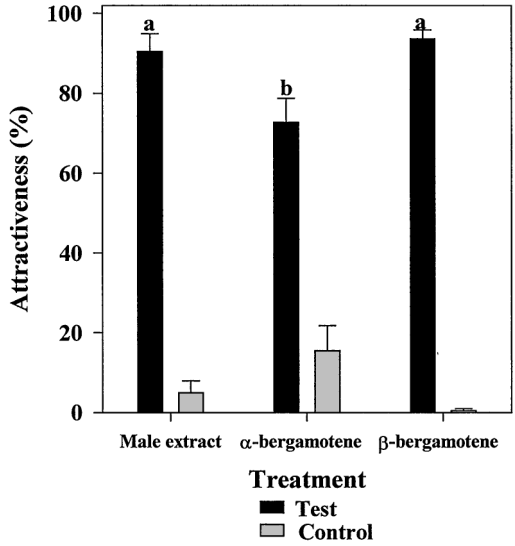


FIG. 4. Response (%) of *M. digitata* females to the synthetic isomers of *trans*-bergamotene identified in the male pheromone blend (Bars followed by the different letters are significantly different by the SNK method, $P < 0.05$; Kruskal-Wallis: $H = 13.67$; $df = 2$; $P = 0.001$, $N = 22$).

at 15°C/min, to 185°C at 5°C/min, to 225°C at 20°C/min): retention time of synthetic α-*trans*-bergamotene and insect-related compound 1 (Figure 2) was 9.2 min, and synthetic β-*trans*-bergamotene and insect-related compound 4 (Fig. 2) had a retention time of 10.13 min, both in between C_{16} (9.17 min) and C_{17} (10–18 min) standards.

Synthetic α-*trans*- and β-*trans*-bergamotene were both attractive to females of *M. digitata*, but α-bergamotene was not as active as the β isomer (Figure 4). Dilution tests of these compounds and the whole extract of males of *M. digitata* showed no difference in the activity between α- and β-bergamotene, but α-bergamotene was always less active than the male extract (Table 1).

Pheromone Release. There is a great variability in the release of pheromone among males of *M. digitata* of the same age, especially at those ages with the highest release rate (Figure 5). Pheromone release was low soon after male emergence (3.9 ng/2 hr), peaking 18.6 ng/2 hr, two days after emergence, and decreasing to only 4.3 ng/2 hr at the end of their life (Figure 5). Release of the male pheromone of *M. digitata* decreases with aging after peaking at the second day after male emergence (Figure 6A) and follows the changes in the pheromone load (Figure 6B). When comparing the amount of pheromone released (Figure 6A) with the amount of pheromone extracted from tissues of the insect (pheromone

TABLE 1. RESPONSE OF VIRGIN FEMALES OF *Melittobia digitata* TO DILUTIONS OF MALE EXTRACT, α-*trans*-BERGAMOTENE AND β-*trans*-BERGAMOTENE (INITIAL CONCENTRATION = 59 NG)

Sample	Female response (%, mean ± SE)[a]			
	5.9 ng	0.59 ng	0.06 ng	Mean
Male extract	79.3 ± 5.6	66.1 ± 8.6	36.9 ± 8.9	60.8 A
α-*trans*-Bergamotene	60.0 ± 9.1	28.5 ± 9.5	10.8 ± 4.0	33.1 B
β-*trans*-Bergamotene	63.8 ± 8.6	54.6 ± 10.2	25.4 ± 7.0	47.9 AB
Mean	67.7 A	49.7 B	24.4 C	

[a]Means in the same column or row followed by the same letters are not statistically different by using Tukey test ($P < 0.05$) (two-way ANOVA: dilution: $F = 21.4$; $df = 2, 116$; $P < 0.001$; Treatment: $F = 8.6$; $df = 2, 116$; $P < 0.001$; Interaction: $F = 0.5$; $df = 4, 116$; $P = 0.706$).

load, Figure 6B), it was also observed that the pheromone is released soon after synthesis as the male ages. *M. digitata* males 1–4 days-old released 14.8–24.4% of their pheromone load in 2 hr, while older males (6–10 days-old) released 91.6–213.0% of the pheromone they had available (Figure 6A,B), which indicates older males release their pheromone as soon as the pheromone is produced. The β isomer is converted to the α isomer on standing, but the α isomer is always present, indicating lack of regio specificity by the sesquiterpene synthase as noted in previous

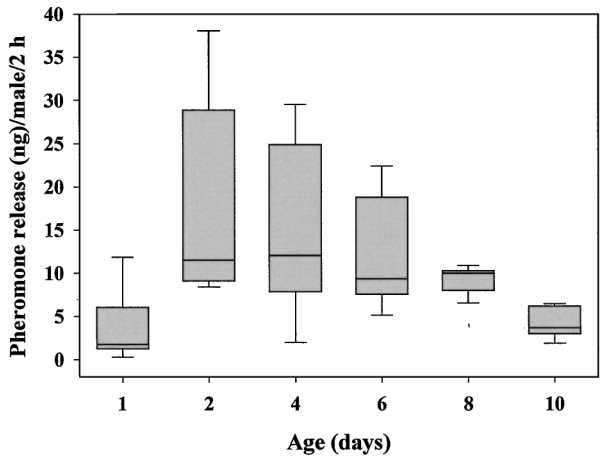


FIG. 5. Pheromone release by *M. digitata* males of different ages. Pheromone is expressed as the sum of α- and β-*trans*-bergamotenes; boxes represent 25th and 75th percentiles, lines within the boxes mark the median, whiskers above and below the boxes indicate the 90th and 10th percentiles.

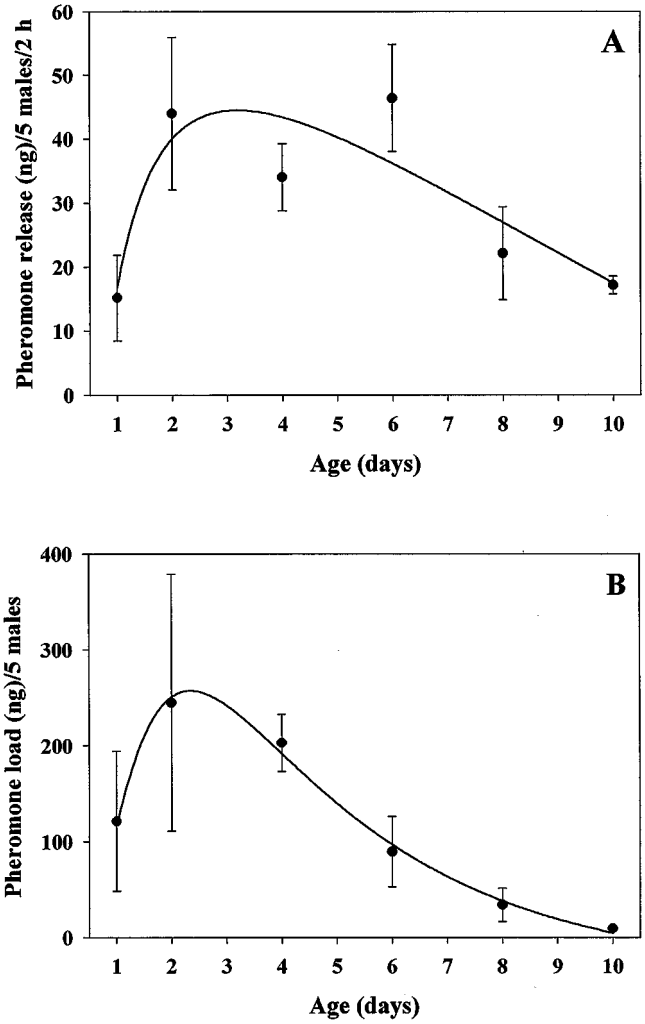


FIG. 6. Pheromone release (A) and pheromone load (B) of males of *M. digitata* of different ages. Pheromone is expressed as the sum of α- and β-*trans*-bergamotenes.

studies of isolated enzyme (Huang et al., 2000; Benedict et al., 2001). The ratio of α- and β-bergamotene remained constant at approximately 5:95 over the lifespan of these males (data not shown).

Although α- and/or β-*trans*-bergamotenes have been reported from a variety of plants, these chemicals are mostly described as minor components of their sesquiterpene fraction (Minyard et al., 1966; Bestmann et al., 1988; Leitão et al.,

1991; Njoroge et al., 1996; Sanda et al., 1998; Pino et al., 2000). They can be found as major components in a few exceptions, such as in the oil of *Lansium anamay-alanum* (Krishnappa and Dev, 1973). However, α-*trans*-bergamotene is the isomer most commonly reported in essential oils from plants, with the β isomer being more rare (Minyard et al., 1966; Bestmann et al., 1988; Flath et al., 1994a,b; Kite and Leon, 1995; Asekun and Ekundayo, 2000). α-*trans*-Bergamotene was also reported as a component of the volatiles released by corn plants in response to the attack of herbivores, but it did not seem to play any role in the response of the third trophic level (Turlings et al., 1991). This chemical has also been identified as one of the components in Angelica seed oil, which is attractive to males of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedmann) (Diptera: Tephritidae) (Flath et al., 1994a). However, further tests demonstrated that α-*trans*-bergamotene was not attractive to males of this fly (Flath et al., 1994b). Furthermore, α-*trans*-bergamotene has also been described as a minor component among the nine volatiles of the male pheromone of the fruit flies, *Anastrepha suspensa* (Loew) and *A. ludens* Loew (Diptera: Tephritidae) (Rocca et al., 1992), but there is no evidence that this component plays any important role in the attractiveness of females of these flies.

This is the first demonstration that males of *M. digitata* produce and release a pheromone attractive to females and the first to chemically identify male-produced pheromone in the parasitic Hymenoptera. β-*trans*-Bergamotene was identified as the main compound of this pheromone, and the β isomer was as active as the whole male extract (Figure 4). The α isomer is also active but, no synergism was noted (data not shown).

Melittobia spp. develop, emerge, and mate within the cocoon of their host. Therefore, the pheromone is probably effective only over a very short range. Matthews et al. (1985) previously discussed the role of the male pheromone as either an aggregation or a sex pheromone and concluded that it would be a sex pheromone in that it appears to function to aggregate and/or arrest virgin females inside the host's cocoon. Mated females and other males do not respond to the male pheromone.

For *M. digitata*, the male pheromone is undoubtedly important for the initiation of courtship, but it may not lead to sex recognition. Rather, the male sex pheromone seems to be the first signal in a series of cues leading to sex recognition and ultimately copulation in this species. Its lack of specificity was suggested by the fact that females would also respond to males of other *Melittobia* species (Matthews et al., 1985).

We have also observed the courtship behavior of *M. digitata*, *M. femorata*, and *M. australica* by exposing virgin females to interspecific males. Although these females were initially attracted to nonconspecific males, they rejected them at very close distances or soon after antennal contact (unpublished data). Males, on the other hand, attempted to copulate with any female, and they engaged in courtship behavior if they managed to mount them. Females avoided interspecific males by

moving away but, in those cases where females were mounted, they restricted antennal contact by folding their antennae against the head (antennal contact is a characteristic of *Melittobia* courtship) or by actively trying to dislodge the males from their backs (unpublished data). These behaviors suggest that *Melittobia* may use at least one other chemical cue for sex recognition, probably a contact or a short-range pheromone.

We have shown that the release of pheromone by *M. digitata* males and the attractiveness of single females to males were quite variable. These results, combined with the fact that females have been shown to prefer certain males over others and that the preference is often maintained in subsequent tests, contribute to the hypothesis that this pheromone can also be used by females as a basis for mate selection. Birkinshaw and Smith (2000) demonstrated that the aggregation pheromone of *Prostephanus truncatus* (Coleoptera: Bostrichidae) was also used during the process of sexual selection. These authors argued that phenotypic variability in pheromone production could be a possible mechanism enabling females to make choices among possible mates.

The male-produced pheromone could also have a role in the fitness of females. Due to their haplodiploid method of sex determination, Godfray (1994) postulated that females of parasitoid wasps would be less likely to invest energy in the production of a sex pheromone since they have the ability to reproduce without insemination. He argued that natural selection should favor the capability of producing pheromones if the production of both sons and daughters would be advantageous. *Melittobia* females fit this prediction and do not invest in sex pheromones. However, *Melittobia* males do allocate part of their energy to pheromone production. Because males are flightless, mating at locations other than the host cocoon is unlikely. Females that disperse to search for hosts without mating would be at a fitness disadvantage compared to mated females competing for a host since female offspring production would be compromised. Thus, a male pheromone that promotes early insemination might be advantageous for females.

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EVIDENCE THAT RIBONUCLEASE ACTIVITY PRESENT IN BEETLE REGURGITANT IS FOUND TO STIMULATE VIRUS RESISTANCE IN PLANTS

RICHARD O. MUSSER,[1,*] SUE M. HUM-MUSSER,[2] SHANNON E. SLATEN-BICKFORD,[3] GARY W. FELTON,[4] and ROSE C. GERGERICH[3]

[1]*Department of Entomology, University of Arkansas, Fayetteville, Arkansas, 72701*

[2]*Department of Horticulture, University of Arkansas, Fayetteville, Arkansas, 72701*

[3]*Department of Plant Pathology, University of Arkansas, Fayetteville, Arkansas, 72701*

[4]*Department of Entomology, Pennsylvania State University, University Park, Pennsylvania, 16802*

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Abstract—*Phaseolus vulgaris* L. cv. 'Pinto' bean is a local lesion host for the plant pathogen Southern bean mosaic virus (SBMV) and its vector is the Mexican bean beetle, *Epilachna varivestis* Mulsant. The objective of this study was to determine if prior feeding by the beetle would affect 'Pinto' bean's resistance to SBMV and determine if ribonuclease (RNase), a major constituent of beetle regurgitant, mediated the plant's response to the virus. 'Pinto' bean plants fed upon by beetles had increased resistance to plant viruses compared to non-wounded or mechanically wounded and buffer-treated plants. Plants that were mechanically wounded and treated with RNase had increased resistance to plant viruses that was equal to plants fed upon by adult beetles. The induction of plant pathogen defenses could be a good adaptation for the plant in the presence of a beetle and pathogen threat. This evidence suggests that RNase activity in the beetle regurgitant could function as an insect-derived elicitor of plant resistance to viruses.

Key Words—Saliva, induced resistance, vector, virus, systemic acquired resistance, elicitor, ribonuclease, RNase, regurgitant.

* To whom correspondence should be addressed. E-mail: rmusser@ag.arizona.edu

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INTRODUCTION

Only recently have characterized oral secretions from insects been determined to alter plant defenses beyond mere mechanical wounding (Mattiacci et al., 1995; Alborn et al., 2000; Musser et al., 2002). Three characterized components of caterpillar oral secretions have been identified that elicit or alter plant defenses, all of which are found in herbivorous caterpillars. These elicitors of plant defenses include β-glucosidase in *Pieris brassicae*, volicitin in *Spodoptera exigua* (Mattiacci et al., 1995; Alborn et al., 2000), and glucose oxidase (GOX) in *Helicoverpa zea* (Felton and Eichenseer, 1999; Eichenseer et al., 1999; Musser et al., 2002).

Regurgitant from insects can stimulate anti-herbivore defenses, plant growth, and phytopathogen resistance (Felton and Eichenseer, 1999). The elicitors β-glucosidase and volicitin have been determined to stimulate anti-herbivore defenses (Mattiacci et al., 1995; Alborn et al., 2000. However, GOX has been found to play a role in the suppression of induced anti-herbivore defenses for *H. zea* (Musser et al., 2002). Tobacco plants fed upon by caterpillars with the ability to secrete saliva had lower levels of nicotine than those tobacco plants fed upon by caterpillars that could not secrete saliva because of cauterized spinnerets (Musser et al., 2002). Glucose oxidase also appears to be an elicitor of plant pathogen defenses. Treatment of wounded soybean plants with GOX reduced the severity of the bacterial disease, *Pseudomonas syringae* pv. *glycinea*, and induced an increase in the phytoalexin daidzein (a marker of systemic acquired resistance) when compared to wounded plants treated with a buffer (Felton and Eichenseer, 1999).

Until now, there was no published evidence that RNases may function as herbivore salivary elicitors of plant defenses. However, exogenous application of RNase increases the resistance of tobacco plants to fungal infection (Galiana et al., 1997). In addition, RNases appear to be induced locally and systemically in wounded *Arabidopsis* plants independent of jasmonate wound signaling as well as salicylic acid found in plant-pathogen defenses (LeBrasseur et al., 2002). RNase has also been shown to play a role in the transmission of plant viruses by beetles (Gergerich et al., 1986). Regurgitant of the Mexican bean beetle, *Epilachna varivestis* Mulsant, contains high levels of RNase (2600 Kalnitsky units; equivalent in activity to 1 mg/ml) (Gergerich et al., 1986). High levels of RNase activity in Mexican bean beetle regurgitant inhibit transmission of non-beetle vectored plant viruses. When these non-beetle transmittable viruses are applied to the beetle in regurgitant during natural feeding, or when regurgitant or RNase is mixed with the viruses and applied to a mechanically wounded plant, the plants do not become infected. Beetle vectored viruses are able to infect plants despite the presence of RNase in beetle regurgitant (Gergerich et al., 1983, 1986). Southern bean mosaic virus (SBMV) is a virus that is vectored by the Mexican bean beetle, and is capable of being transmitted in the presence of RNase in the inocula used in mechanical wounding (Gergerich et al., 1983, 1986). The objective of this study was

to determine whether the RNase that is present in the regurgitant of the Mexican bean beetle might induce systemic resistance to SBMV.

METHODS AND MATERIALS

Plants and Insects. *Phaseolus vulgaris* L. cv. 'Pinto' seeds were sown in 4-L plastic pots containing Redi Earth Peat-Lite Mix, a potting soil mixture (Scott-Sierra Horticulture Products Company, Marysville, OH). Plants were grown in a greenhouse with a 14-hr photoperiod, using natural light conditions and high pressure sodium lights (1000 W), and day and night temperatures between 33°C and 25°C. Plants were watered daily and fertilized weekly (N:P:K = 15:30:15, American Plant Food Company, Creve Coeur, MO). Mexican bean beetles were reared from eggs obtained from Charles Helms (University of Illinois). The beetle colony was maintained on 'Pinto' bean, a local lesion host for SBMV.

Viruses. SBMV was maintained in 'Black Valentine' bean, a systemic host for this virus. Approximately once every two weeks, virus was transferred by mechanical rub-inoculation to healthy seedlings that had been dusted with carborundum. Purification of SBMV from 'Black Valentine' bean was performed in a manner described by Gergerich et al. (1983).

Beetle Feeding Experiment to Test Plant Susceptibility to Virus Infection. Seedling 'Pinto' bean plants (9 days old) with fully expanded primary leaves were fed upon by a single adult Mexican bean beetle caged on one of the primary leaves for 24 hr. The clip cage composed of two, 4-cm diameter medicine-cups, held together by a hair clip. 'Pinto' bean plants were also mechanically wounded with a 4-cm^2 garlic press on one primary leaf. Half of these mechanically wounded plants were treated with 0.01 M potassium phosphate buffer, pH 7.0; the other half were treated with 1 mg/ml ribonuclease A (RNase A, Qiagen Valencia, CA) diluted in the buffer. Mexican bean beetle regurgitant contains RNase activity and substrate specificity similar to 1 mg/ml of RNase A (Gergerich et al., 1986). The mechanically wounded area was caged with a clip cage. As a control treatment, non-wounded plants had a clip cage attached to one primary leaf. After 24 hr, the cages and beetles were removed and plants were given a 48-hr recovery period. The opposite seedling leaf was then mechanically inoculated with SBMV in the manner described above. The leaf was inoculated with a 10^{-4} dilution of a purified SBMV preparation (22 mg/ml) in 0.01 M potassium buffer, pH 7.0. Five days later, the local lesions on the inoculated leaf were counted. To aid in the consistency of lesion counting between leaves, the lesions were counted in three 1-cm^2 squares directly adjacent to the mid-vein. Each replication consisted of ten random plants for treatment with seven replications. The mean lesion number for the experiment was compared with a Fisher's least significant differences (LSD) test in a one-way ANOVA with JMP.

RESULTS AND DISCUSSION

'Pinto' bean seedlings that were fed upon by the Mexican bean beetle, or mechanically wounded and treated with RNase had significantly fewer local lesions (>20% less) than non-wounded and mechanically-wounded plants without RNase (Fisher's LSD; Figure 1). Non-wounded and mechanically-wounded plants had similar levels of local lesions and were not significantly different from each other; but there was a trend towards more local lesions for the non-wounded bean plants. However, this is not the first example of beetle feeding reducing infection rates in plants. Beetle feeding on *Rumex obtusifolius* has been shown to reduce the rate of infection by fungal pathogens (Hatcher and Paul, 2000).

'Pinto' bean plants are systemically more resistant to SBMV following Mexican bean beetle feeding as indicated by a reduction in local lesions on bean leaves. Upon infection of SBMV or bean pod mottle virus (BPMV), the 'Black Valentine' cultivar may develop whole plant infection, while the 'Pinto' cultivar only forms local lesions. In general, the difference between the pathogen-resistant cultivars and non-resistant cultivars appears to be due to the speed and strength of induction of the systemic acquired resistance pathway (SAR) (Morris et al., 1998). However, additional experiments have demonstrated that beetle feeding increases systemic resistance for BPMV in 'Black Valentine' bean plants (Musser,

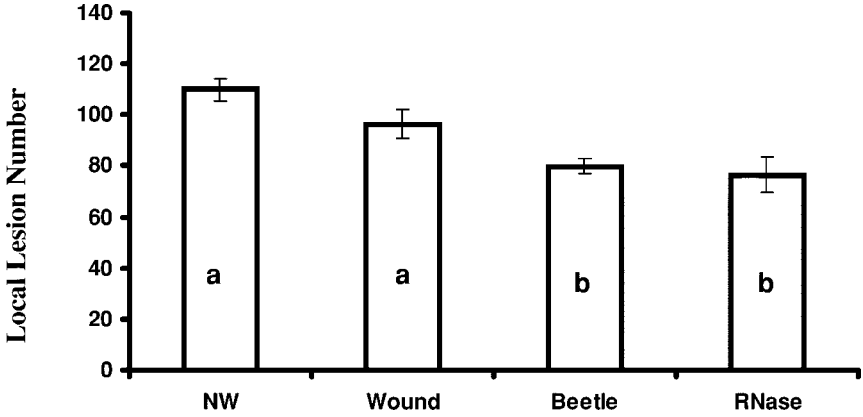


FIG. 1. Reaction of 'Pinto' bean plants to SBMV inoculation after beetle feeding or treatment of mechanical wounds treated with RNase or buffer. Lesion number is the total number of local lesions in three 1-cm^2 squares on the inoculated leaf. Different letters represent statistically significant differences between treatments at $P < 0.05$. Bars indicate mean $\pm$ s.e. Legend: NW = untreated bean plants, Wound = mechanically wounded leaf treated with buffer, Beetle = leaf fed upon by beetle, and RNase = mechanically wounded leaf treated with RNase.

unpublished data). Although the bean plant exhibits systemic resistance to the beetle vectored virus, it does not appear that RNase is linked to SAR formation (LeBrasseur et al., 2002).

Ribonuclease activity is found in the Mexican bean beetle regurgitant at high levels (Gergerich et al., 1983, 1986). 'Pinto' bean leaves that were wounded and treated with RNase had stimulated virus resistance that was active at least three days after wounding. This evidence suggests that RNase activity in the regurgitant of the Mexican bean beetle functions as an elicitor of plant pathogen defenses. For bean plants, insect feeding and RNase could be a reliable predictor of impending pathogen infections. From an evolutionary perspective, this could be considered a good adaptation for the bean plant to increase resistance to virus infection when confronted by an insect and pathogen threat. Interestingly, the RNase present in beetle regurgitant has been shown to inhibit infection of most plant viruses when the viruses are deposited by the beetle in regurgitant during feeding (Gergerich et al., 1983, 1986). Thus, the induction of resistance on a systemic level can be regarded as a second line of defenses against those viruses that are beetle transmissible, i.e., the viruses that are able to infect a plant in spite of the presence of RNase in beetle regurgitant.

Ribonuclease found in the beetle regurgitant appears to have elicitor qualities in plant resistance to viruses. However, it has yet to be determined if RNase activity in the beetle regurgitant effects anti-herbivore defenses of plants, and what function RNase activity in the regurgitant has for the beetle. This study further demonstrates the need to understand the biochemical constituents of insect oral secretions in the elicitation of induced plant defenses.

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DO EURASIAN BEAVERS SMEAR THEIR PELAGE WITH CASTOREUM AND ANAL GLAND SECRETION?

FRANK ROSELL*

Faculty of Arts and Sciences, Department of Environmental and Health Studies, Telemark University College, N-3800 Bø in Telemark, Norway

Department of Zoology, Norwegian University of Science and Technology, N-7491 Trondheim, Norway

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Abstract—The scent-matching hypothesis postulates that scent marks provide an olfactory link between a resident owner and his territory, and that this enables intruding animals to recognize the chance of escalated conflicts. However, it is unclear if Eurasian beavers (*Castor fiber*) mark their own pelage with castoreum from their castor sacs (i.e., the same material used in territorial marking); and/or if beavers mark their pelage with anal gland secretion (AGS) from the anal glands to waterproof the pelage and to act as a "living-scent mark." Chemical analysis (gas chromatography and mass spectrometry) of hair samples from 22 live-trapped beavers revealed that castoreum compounds were not present in any samples, AGS compounds were found from 3 animals (13.6%) around the cloaca, and the compound squalene was found in all the samples. Beavers may release castoreum directly into the water when it meets an intruder. Thereby, the "scent mark" in the water can provide an olfactory link between a resident owner and his territory. Squalene, in contrast to AGS, may be essential for keeping beaver pelts water-repellant.

Key Words—Beaver, *Castor fiber*, anal gland secretion, castor sacs, castoreum, pelage, GC-MS, hair, squalene, Norway.

INTRODUCTION

The scent-matching hypothesis postulates that scent marks provide an olfactory link between a resident owner and his territory, and that this enables intruding animals to recognize the chance of escalated conflicts. If the smells match, then the animal that the intruder has met must be the territory owner. If the hypothesis were

* To whom correspondence should be addressed. E-mail: Frank.Rosell@hit.no

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true, one would expect owners to (1) mark where intruders are most likely to encounter marks; (2) mark themselves with the substances used to mark the territory; (3) make themselves available for scent matching by intruders; and (4) remove or replace marks of others (Gosling, 1982).

All family members of Eurasian beavers (*Castor fiber*), except kits less than five months old, participate in marking the territory at scent mounds close to the water's edge (Wilsson, 1971; Rosell, 2002). Predictions 1, 3, and 4 for the scent-matching hypothesis have all received some support for the Eurasian beaver (Rosell, 2002). However, prediction 2 needs to be further clarified. Castoreum (primarily a mixture of secondary metabolites from urine) i) is the main scent signal used in the defense of beaver territories during winter (Rosell and Sundsdal, 2001). However, the anal gland secretion (AGS) may function to waterproof the pelage. Excision of the anal glands seems to reduce the ability of the pelage to repel water (Walro and Svendsen, 1982). Prohibition of autogrooming in Eurasian beaver produces similar results (Wilsson, 1971). AGS may also act as a chemical messenger in the water territory sensed through close range or contact with the animal (Rosell, 2002).

The aim of this study was to investigate and to search for characteristic chemical compounds from the castor sacs and the anal glands in the beaver pelage in an attempt to clarify if (1) beavers mark themselves with castoreum from their castor sacs i.e., the same material used in territorial marking; and/or if (2) beavers mark themselves with AGS from the anal glands to waterproof the pelage and to act as a "living-scent mark."

METHODS AND MATERIALS

Study Area and Animals. Twenty-two beavers were live-trapped (15 males and 7 females) from 9 different territories during June 2 to August 18, 2000, by using landing nets in the municipalities of Nome and Sauherad in Telemark County, Norway. Beavers were from different age-classes (1−>6 years old), weight varied from 3–24.5 kg, and they were trapped between 2100–0230 hr (see Rosell and Hovde, 2001 for further details about handling, sex, and age determination).

Sample Collection. Hair samples were collected from 3 different places on the beavers: 1) at the back just above the scaly tail, 2) around the cloaca, and 3) on the belly. Samples were collected using clean scissors. I cut off hair onto a sheet and thereafter stored it in a 100 ml glass vial. After each sampling, the scissors were cleaned with alcohol and dried with a propane gas burner.

I collected castor sacs and anal glands from 60 animals shot locally during the normal hunting season from 28 January–6 May 1997–99 and used the castoreum and AGS from these animals as the basis for comparison with possible castoreum and AGS compounds present in the hair samples. See Rosell and Sundsdal (2001)

for a review of types of compounds found in castoreum and AGS. All samples were stored at -20°C until chemically analyzed (for further details see Rosell and Sundsdal, 2001).

Sample Preparation. Hair samples (0.15 g) were extracted overnight in 10 ml toluene. The solutions were filtered in a glass funnel with Schleicher & Schuell 595 paper filter. Thereafter, the hair was squeezed as dry as possible with a glass rod on the filter. The solutions were evaporated to a volume of 0.25 ml in a Heidolph VV 2011 rotary evaporator to concentrate the solution. See Rosell and Sundsdal (2001) for preparation of AGS and castoreum.

Chemical Analysis. One μl of the resulting solution was injected into a Hewlett-Packard 6890 Series II gas chromatograph equipped with a nonpolar HP-5 MS 5% phenyl-methyl-siloxane column (30.0 m $\times$ 0.25 mm ID $\times$ 0.25 μm film thickness) installed in a Hewlett-Packard 5973 Series mass spectrometer detector with a split/splitless inlet used in the splitless mode (for further details see Rosell and Sundsdal, 2001). The main focus of my study was to determine if the beavers had castoreum and/or AGS in their pelage. Therefore, positive identification of the compounds was not attempted, i.e., comparison with a known standard was not done. However, the compound squalene was positively identified (see results/ and discussion). Rosell and Sundsdal (2001) showed that this method worked well to detect compounds from the castoreum and the AGS in scent marks on snow.

RESULTS AND DISCUSSION

I found no compounds from the castor sacs on the pelage, which suggests that beavers do not mark themselves with castoreum. However, it is well known that beavers urinate into the water, i.e., release castoreum (Wilsson, 1971). They also release castoreum when frightened, e.g., when captured and handled (personal observation). Beavers may have adopted an alternative strategy, i.e., they may release castoreum directly into the water when they meet an intruder. Thereby, the "scent mark" in the water can provide an olfactory link between a resident owner and his territory, and this will enable an intruding animal to gauge the chance of escalated conflicts.

Compounds from the anal glands were found in 3 of 22 animals (13.6%) but only around the cloaca area (1 (male, 3 years old), 8 (male, >5 years old) and 19 (female, 4 years old) compounds, respectively). This suggests that beavers do neither mark themselves with AGS to waterproof the pelage, nor act as a "living scent mark." Wilsson (1971) was not able to observe that the beaver systematically touched the opening of the anal glands or the area around these during grooming. For the Eurasian beaver, the AGS is a thick paste of a greyish color in females, and is an oily fluid with a whitish or pale straw color in males. For the North American beaver (*C. canadensis*), the AGS of the two sexes show also a consistent difference

regardless of age: it is brown and viscous in males, but it is whitish or light yellow and runny in females (see Rosell and Sun, 1999). Obviously, the viscous secretion of the Eurasian female and the North American males would make the pelage sticky and would lessen water repellency of the pelage. The differences in viscosity between the sexes make it also unlikely that the anal glands have been developed to waterproof the pelage.

The sebaceous glands in the skin of mammals produce lipid mixtures (sebum) which coat the skin and hair. A relative minor contribution to this lipid film is made by the lipids arising from the keratinized cells of the epidermis (Lindholm et al., 1981). The results from this study showed that the hydrocarbon squalene ($C_{30}H_{50}$) was positively identified in all the hair samples. Squalene is also found in the skin surface lipids of the North American beaver (Lindholm and Downing, 1980). Therefore, when beavers groom, the pelage is cleaned, the hair shaft is aligned, maintaining maximum loft, and the hairs are probably oiled with the aid of squalene, which reduces water permeability. This could explain why prohibition of autogrooming in Eurasian beaver reduced the ability of the pelage to repel water (Wilsson, 1971). In addition to the natural lipid squalene, the density of the underfur (23 000 cm^{-2} on the ventral and 12 000 cm^{-2} on the dorsal surface), the guard hairs, and the surface tension of the water help to prevent water from penetrating to the skin (Novak, 1987). Thus, squalene, in contrast to AGS, may be essential for keeping beaver pelts water-repellant.

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PLANT-TO-PLANT COMMUNICATION MEDIATING IN-FLIGHT ORIENTATION OF *Aphidius ervi*

E. GUERRIERI,[1] G. M. POPPY,[2] W. POWELL,[3]
R. RAO,[4] and F. PENNACCHIO[5,*]

[1]*Istituto parla Protezione delle Piante CNR Sez. di Portici*
via Università 133, 80055—Portici (NA), Italy

[2]*School of Biological Sciences, University of Southampton*
Bassett Crescent East, Southampton, Hants SO16 7PX, United Kingdom

[3]*IACR—Rothamsted*
Harpenden, Hertfordshire AL52JQ, United Kingdom

[4]*Dipartimento di Scienze del Suolo, della Pianta e dell'Ambiente*
Università di Napoli "Federico II"
via Università 100, 80055—Portici (NA), Italy

[5]*Dipartimento di Biologia, Difesa e Biotecnologie Agro-Forestali*
Università della Basilicata
Macchia Romana, 85100—Potenza, Italy

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Abstract—Broad bean plants (*Vicia faba*) infested by the pea aphid, *Acyrthosiphon pisum*, play a key role in the in-flight orientation of the parasitoid *Aphidius ervi*, by producing host-induced synomones (HIS). These volatiles are herbivore-specific and are systemically released from insect-free parts of an infested plant, suggesting the existence of an elicitor circulating throughout the plant. This study was designed to investigate whether the plant metabolic changes, leading to HIS biosynthesis and emission, can in some way trigger similar responses in neighboring plants through aerial and/or root communication. Uninfested broad bean plants maintained in the same pot together with plants infested by *A. pisum* became more attractive towards *A. ervi* females when tested in a wind-tunnel bioassay. This change was not observed when root contact was prevented among plants that had their aerial parts in close proximity, suggesting that an exudate from the roots of the infested plant may cause the induction of the attractive volatiles in uninfested plants. Broad bean plants grown hydroponically also produce pea aphid induced signals that attract *A. ervi*. When an intact (uninfested) plant was placed in a hydroponic solution previously used to grow a pea aphid-infested plant, it became attractive to parasitoids, while an intact plant placed in a solution previously used to grow an intact plant did not undergo such a change.

* To whom correspondence should be addressed. E-mail: pennacchio@unibas.it

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These results indicate that plant-to-plant signaling in this tritrophic system may occur at the rhizosphere level and is most likely mediated by a systemically translocated elicitor.

Key Words—Synomones, phytopheromones, wind tunnel, flight behavior, Aphidiinae, Aphididae.

INTRODUCTION

Plants use different ways of defending themselves against herbivorous arthropods. Recruiting natural enemies of herbivores by releasing attractive volatiles appears to be a widespread phenomenon that has been referred to as indirect defense to distinguish it from the direct defense that a plant displays in the form of structures or metabolites reducing or inhibiting herbivore attack (Dicke, 1999). In most cases, the emission of attractive volatiles is triggered by the feeding activity of herbivores. These volatiles are commonly referred to as herbivore-induced synomones (HIS), to stress the mutual benefit gained by both plants and natural enemies (see Vet and Dicke, 1992 for detailed references), although some scientists remain sceptical about the real benefits obtained by the plant (Meijden and Klinkhamer, 2000).

A considerable degree of blend specificity has been convincingly demonstrated in a number of tritrophic systems (for review see Dicke and van Loon, 2000). Furthermore, in many cases, it has been shown that these volatiles are systemically emitted by the plant, and tissue damage at the emission site is not required (Turlings and Tumlinson, 1992; Dicke, 1994; Potting et al., 1995; Röse et al., 1996). The volatile blends emitted in response to herbivore feeding are often different from those induced by mechanical damage (Turlings et al., 1995; Takabayashi and Dicke, 1996). Herbivore oral secretions have been shown to elicit this specific plant reaction when applied to mechanically damaged plant tissues or systemically delivered to the plant (for review see Dicke and van Loon, 2000). The role of these elicitors in activating the signal transduction pathways triggering the biosynthesis of HIS has been partly elucidated and, to date, two herbivore-derived elicitors have been identified from two different caterpillar species (Mattiacci et al., 1995; Alborn et al., 1997; for review see Walling, 2000). However, the elucidation of the ecological role of these substances within complex natural food webs is still in its infancy.

The foraging behavior of the aphid parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae) has been investigated intensively in recent years, elucidating the role of semiochemicals associated with both first and second trophic levels (Guerrieri et al., 1993, 1997, 1999; Du et al., 1996, 1997, 1998; Powell et al., 1998). It has been demonstrated that the plant plays a key role in the in-flight orientation of this parasitoid by emitting HIS (Guerrieri et al., 1993; Du et al.,

1996). Furthermore, these volatiles are herbivore-specific and are produced when a certain threshold of infestation, in terms of number of aphids and hours of feeding activity, has been reached (Du et al., 1996; Guerrieri et al., 1999). Finally, it has also been demonstrated in this system that the synomones are systemically released from insect-free parts of the infested plant, suggesting the existence of an elicitor circulating throughout the plant (Guerrieri et al., 1999).

This study addresses whether the plant metabolic changes leading to HIS biosynthesis and emission can in some way trigger similar responses in neighboring plants through aerial and/or root communication. Plant-to-plant communication remains a difficult issue to deal with (Chamberlain et al., 2001), especially in the context of phytopheromones (Pickett and Poppy, 2001), but tritrophic systems and their complexity may offer interesting experimental opportunities to analyze this intriguing phenomenon from a new perspective.

METHODS AND MATERIALS

Experimental Animals

A. ervi was reared on its natural host *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae), maintained on potted broad bean plants (*Vicia faba* L., Angiospermae: Leguminosae, cv. Aquadulce). Aphid and parasitoid cultures were kept in separate environmental chambers at $20 \pm 1^{\circ}$C, $65 \pm 5\%$ relative humidity, 18L:6D photoperiod. The parasitoid culture was periodically refreshed by adding adults emerging from field collections of *A. pisum* mummies.

Insect parasitoids used in the bioassays were reared as synchronized cohorts by exposing heavily infested plants for 24 hr to 1-day-old, mated females; after a week the resultant mummies were clipped from the plant and isolated in glass test tubes (60×8 mm) plugged with cotton-wool. Experimental females were mated and fed with a 50% honey solution and used between the first and the second day after emergence. All experiments were conducted between the third and the seventh hr from the onset of the photophase.

Wind Tunnel

Behavioral observations were carried out in a wind tunnel similar to that described by Miller and Roelofs (1978), modified according to Guerrieri et al. (1993). The wind speed, monitored with a hot-wire anemometer (Alnor, model CGA-26), was adjusted to 25 ± 1 cm/sec in all experiments. The exhausted air was vented to outside the wind-tunnel room. The light intensity in the flight chamber was around 3600 lux at the take-off point. A black and white striped pattern was placed beneath the flight chamber floor to aid visual orientation.

Bioassay

All the experiments were carried out at $20 \pm 1^{\circ}$C, $65 \pm 5\%$ relative humidity. Individual parasitoids were used only once and were released from an open-ended glass test tube held on a plastic base so that the take-off point was exactly in the middle of a cross section of the flight chamber. Fifty to 75 parasitoid females were tested for each target by releasing them individually into the odor plume 30 cm downwind from the target. Parasitoids were observed for a maximum time of 10 min, and flight behavior data were recorded and analyzed with the aid of event-recording software (The Observer, Noldus Information Technology, Wageningen, The Netherlands). A flight response was considered as oriented towards the experimental target when the female flew directly upwind and landed on or not further than 5 cm away from the target. Experiments were conducted on several days and targets were presented in a random order each day to reduce the effect of any temporal variability in responsiveness.

Soil-Based Experiments

Experiment I: Rhizosphere-Transfer. Four 7-day-old broad bean plants were potted together in sterile soil in a 15-cm-diam plastic pot and, after 2 days, two of them were infested with several hundred *A. pisum*. Aphids were prevented from moving onto uninfested plants by means of a cylindrical cage placed over infested plants. The cage was made from an overhead transparency sheet (A4 size) with the top and a 15×10-cm lateral window covered with anti-insect net. This allowed the passage of light and the normal circulation of air to prevent the wilting of infested plants. After 5 days, the infested plants were gently removed from the pot and the uninfested plants (referred to as PHC-pot-conditioned, where PHC = plant–host complex) were immediately tested in a no-choice wind-tunnel bioassay. Four uninfested plants were prepared as described above, and two of them were caged but not infested and eventually removed after 5 days while the remaining two acted as a control (referred to as P-pot-conditioned, where P = uninfested plant).

Experiment II: Aerial Transfer. Seven-day-old broad bean plants were potted in pairs in sterile soil. After 2 days, some of them were infested with several hundred *A. pisum* and surrounded by pots containing uninfested plants. Aphids were prevented from moving onto uninfested plants as described in experiment I. The aerial parts of the neighboring treatment plants (infested and uninfested) were separated by the same distance as in experiment I, but their roots were now confined in separate pots. After 5 days, uninfested plants that had been in close aerial contact with infested plants (referred to as PHC-air-conditioned) were tested in a no-choice wind-tunnel bioassay. Uninfested plants grown close to other uninfested plants caged as described above (referred to as P-air-conditioned) acted as controls.

Hydroponic Experiments

Hydroponic Plant Culturing. Broad bean seeds (cultivar Aquadulce) were potted in sterile soil and kept in a glasshouse at 20°C. After 10 days, the seedlings were gently removed from the soil, the cotyledons peeled and the roots rinsed with bidistilled water, carefully brushing away all soil particles. They were then placed in glass beakers containing a hydroponic solution made with Murashige and Skoog basal salt mixture (referred to as MS hereafter) (Sigma, M5524), 2 g/liter, and maintained in an environmental chamber at 20°C. Each beaker, containing two seedlings, was wrapped in aluminum foil to hold the plants in position and to prevent the light from reaching the roots. Every 2 days, the hydroponic solution in each beaker was renewed. These were used as the starting plants for experiments III and IV.

Experiment III: Effects of Hydroponic Culture on Plant Signaling. The hydroponic solution was replaced after 2 days, and some of the plants were infested with several hundred *A. pisum*. After 3 days, uninfested and infested plants were used in a no-choice wind-tunnel bioassay to test their attractiveness to *A. ervi* females.

Experiment IV: Hyrdoponic Transfer of an Elicitor. Infested and uninfested plants were obtained as described for experiment III, but after 3 days, all of them were removed from their beakers and substituted with new uninfested plants. After 24 hr, plants conditioned by hydroponic solution from uninfested or infested plants (referred to as MS-P–conditioned and MS-PHC–conditioned, respectively) were used in a no choice wind-tunnel bioassay.

Statistical Analyses

The number of parasitoids responding to each target in any experiment was compared by a G test for independence with William's correction (Sokal and Rohlf, 1995). The resulting values of G were compared with the critical values of χ^2 (Rohlf and Sokal, 1995).

RESULTS

Experiment I: Rhizosphere Transfer. This experiment was designed to assess whether uninfested plants could be conditioned to produce attractive volatiles when planted together with infested plants in the same pot. Uninfested plants that had been potted together with infested plants (PHC-pot-conditioned) were significantly more attractive than control plants (P-pot-conditioned) ($\chi^2 = 14.48$; $df = 1$; $P < 0.001$ for oriented flights; $\chi^2 = 10.68$; $df = 1$; $P < 0.005$ for landings on the target) (Figure 1). In fact, 88% of tested *A. ervi* females showed oriented flight towards PHC-pot-conditioned plants and 84% landed on the target, compared with 54%, in both cases, against P-pot-conditioned plants.

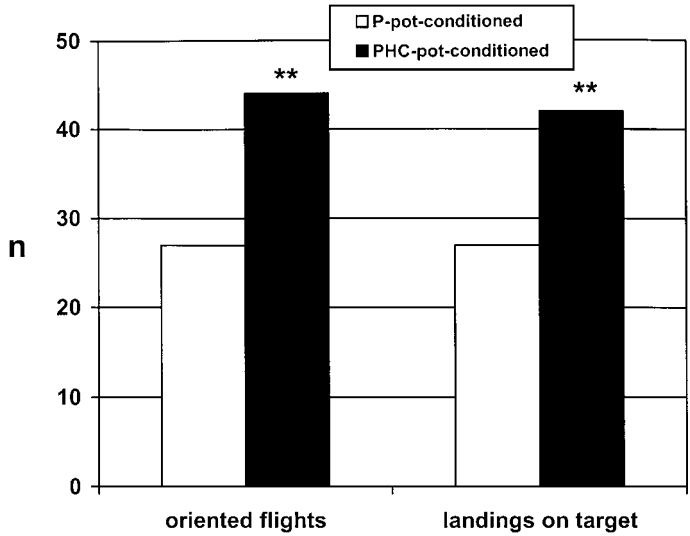


FIG. 1. Flight response of *Aphidius ervi* females towards two uninfested plants that were potted together with either two uninfested (P-pot) or two infested (PHC-pot) plants. In the latter case, aphids were prevented from moving onto uninfested plants by means of cylindrical cages made of anti-insect net. After removing the two uninfested or infested plants, the remaining two, always uninfested, were used as targets in wind-tunnel bioassays. A total of 10 plants (five pairs) and 50 female parasitoids were used for each test on four consecutive days. Significant differences within each behavioral response category are denoted ($^{**}P = 0.001$).

Experiment II: Aerial Transfer. This experiment was designed to assess whether the volatiles produced by infested plants could induce uninfested plants placed nearby, but planted in different pots, to produce volatiles attractive to *A. ervi* females. Uninfested plants placed close to separately potted, infested plants (PHC-air-conditioned) elicited similar flight responses and landings on the target by *A. ervi* females as those recorded for control plants (P-air-conditioned) ($\chi^2 = 0.059$; $df = 1$; $P < 0.9$) (Figure 2).

Experiment III: Effects of Hydroponic Culturing on Plant Signaling. This experiment was designed to assess whether the use of a hydroponic solution as a growing medium would affect the flight behavior of *A. ervi*, in response to volatile plant and host stimuli. There were significant differences between the percentages of oriented flights and landings on the target by *A. ervi* females recorded for MS-P (36%, 32%) and MS-PHC (70%, 56%) ($\chi^2 = 11.68$, $df = 1$; $P < 0.001$ for oriented flights; $\chi^2 = 5.83$; $df = 1$; $P < 0.025$ for landings on the target), consistent with results previously recorded for soil grown plants (Guerrieri et al., 1993). (Figure 3).

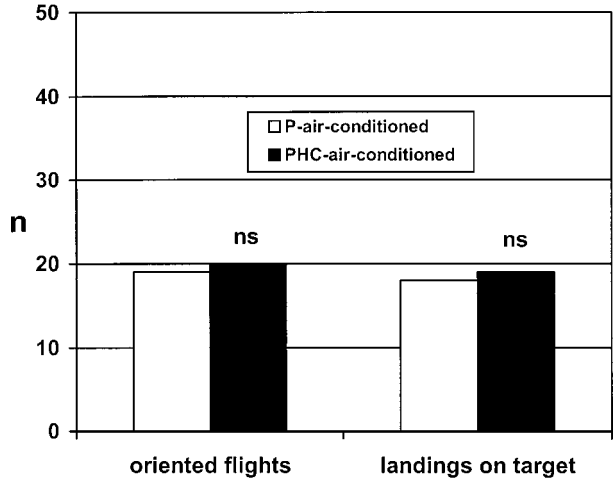


FIG. 2. Flight response of *Aphidius ervi* females towards two uninfested plants potted together and placed in close proximity to either two uninfested (P-air) or two infested (PHC-air) plants. The experimental plan was similar to that described in Figure 1, but root contact was prevented by keeping the plants in different pots, while their aerial parts were maintained at the same distance as in the previous experiment. A total of 10 plants (five pairs) and 50 female parasitoids were used for each test on four consecutive days. There were no significant differences, within each behavioral response category.

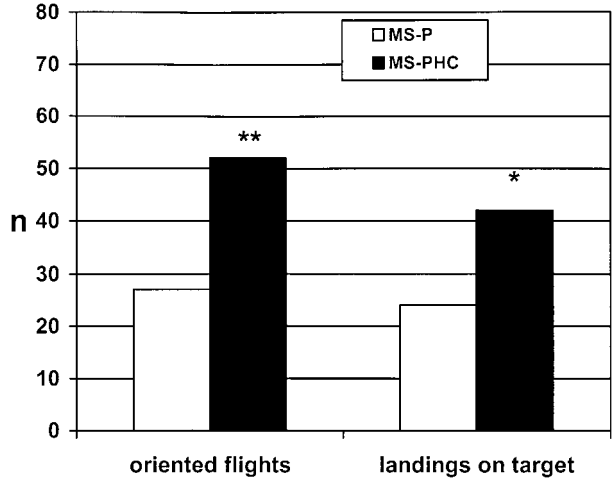


FIG. 3. Flight response of *Aphidius ervi* females towards two uninfested (MS-P) or two infested plants (MS-PHC) grown in hydroponic solution. A total of 10 plants (five pairs) and 75 female parasitoids were used for each test on four consecutive days. Significant differences within each behavioral response category are denoted ($^{*}P = 0.05$; $^{**}P = 0.001$).

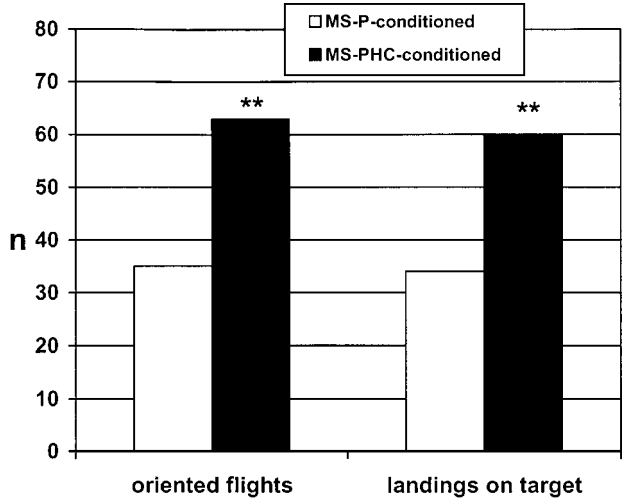


FIG. 4. Flight response of *Aphidius ervi* females towards two uninfested plants conditioned by keeping them for 24 hr in a hydroponic solution previously used to grow either two uninfested (MS-P) or two infested (MS-PHC) plants for three days. A total of 10 plants (five pairs) and 75 female parasitoids were used for each test on four consecutive days. Significant differences within each behavioral response category are denoted ($^{**}P = 0.001$).

Experiment IV: Hydroponic Transfer of an Elicitor. This experiment was designed to assess whether the compounds released by infested plants into the hydroponic solution could induce uninfested plants to emit volatiles attractive to *A. ervi* females. Uninfested plants that had been maintained for 24 hr in a hydroponic solution previously occupied by infested plants (MS-PHC-conditioned) were significantly more attractive than control plants (MS-P-conditioned) to *A. ervi* females (Figure 4). In fact, MS-PHC-conditioned plants elicited more oriented flights (84%) and landings on the target (80%) than MS-P-conditioned plants (46.7% and 45.3%, respectively)($\chi^2 = 19.79$; $df = 1$; $P < 0.001$ for oriented flights; $\chi^2 = 19.60$; $df = 1$; $P < 0.001$ for landings on the target).

DISCUSSION

Previous studies on the flight behavior of *A. ervi* have shown that broad bean plants emit volatiles attractive to this parasitoid as a consequence of infestation by its host *A. pisum* (Guerrieri et al., 1993; Du et al., 1996). Furthermore, these volatiles are released from insect-free parts of infested plants, suggesting either the existence of compounds (elicitors) circulating throughout the plant and regulating

these emissions or, more unlikely, movement of the attractive compounds themselves throughout the plant (Guerrieri et al., 1999).

The present study demonstrates that some compounds responsible for the production of aphid-induced plant volatiles, to which parasitoids are attracted, can be exchanged below ground between adjacent plants via root exudates, in the system *V. faba–A. pisum–A. ervi*. Uninfested plants, placed for some days with their roots and air-space contiguous with those of infested plants, became as attractive to parasitoid females as the infested plants, eliciting significantly more oriented flights and landings on the target compared to control plants. There could be two explanations for this phenomenon. Either an elicitor is released into the rhizosphere or air, which acts like a phytopheromone, causing neighboring plants to produce volatiles that attract parasitoids, or the attractive volatiles are adsorbed onto neighboring plants, which then re-release the volatiles. However, this enhancement of attraction was not evident when the root contact was prevented (experiment II), suggesting that aerial transfer of an elicitor or aerial adsorption of attractive volatiles and their subsequent re-release would not explain this phenomenon. In fact, uninfested plants placed in close proximity to infested plants but in separate pots, elicited similar percentages of oriented flights and landings on the target as control plants, and these values were not different from those recorded for uninfested plants of the same age, which were grown without any kind of conditioning (Guerrieri et al., 1993, 1999). However, an increased attractiveness of plants towards parasitoids can be obtained through aerial conditioning of uninfested plants by using a volatile phytopheromone. For example, intact broad bean plants placed for 24 hr in a glass vessel in the presence of (*Z*)-jasmone, became as attractive to *A. ervi* in wind-tunnel tests as infested plants. This was thought to be due to the increase in the production of (*E*)-β-ocimene, known to attract other parasitoids (Birkett et al., 2000). However, these experiments used synthetic *cis*-jasmone at concentrations higher than that normally released by damaged plants, and, thus, a conclusive demonstration of a phytopheromone still remains elusive for scientists working with plant signalling (Pickett and Poppy, 2001).

The presence of compound(s) exchanged between plants via the rhizosphere was confirmed by experiment IV, in which an infested plant incubated for 24 hr in a hydroponic solution recently vacated by an infested plant became as attractive as the infested plant for *A. ervi* females, in terms of both oriented flights and landings on target, and significantly more attractive than control plants. Control plants placed in hydroponic solution showed poor attractiveness, compared with aphid infested plants, as previously reported for soil-grown plants (Guerrieri et al., 1993; Du et al., 1996).

The combined results of our tests with soil-grown and hydroponic-grown systems show that below-ground communication between adjacent plants can influence above-ground emission of volatiles that regulate tritrophic interactions.

However, a possible role of aerial transfer of a signal or direct adsorption of attractive volatiles cannot be totally ruled out, even though its importance appears to be marginal in our test system.

In another tritrophic system, a detached leaf of lima bean incubated for 3 days with its petiole in the water where a leaf infested with the spider mite *Tetranychus urticae* Koch had been previously placed for 7 days became as attractive as infested leaves to the predatory mite *Phytoseiulus persimilis* (Athias-Henriot), when tested in a Y-tube olfactometer (Dicke et al., 1993). More recently, these results were confirmed by using an elicitor collected from whole lima bean plants that had only one primary leaf infested with spider mites (Dicke and Dijkman, 2001). Furthermore, the leaves coming from an uninfested plant incubated for 3 days in the water where an infested plant had previously been reared for 7 days became, in a Y-tube olfactometer, as attractive to *P. persimilis* as infested leaves and significantly more attractive than control leaves.

Even though Dicke's research differs from that reported in the present paper in terms of the plant material used (detached leaves versus whole plants), the methods used (Y-tube olfactometer versus wind tunnel), and principally in the natural enemy studied (predator versus parasitoid), the results are for the most part highly comparable (Dicke et al., 1993; Guerrieri et al., 1999). However, in the spider mite–predator system, the required time of exposure of the uninfested plant to the elicitor is longer than that evident for the hydroponic experiment in our study, in which an exposure of only 24 hr was sufficient to make a whole uninfested broad bean plant attractive to *A. ervi*. This difference could be a reasonable consequence of the different plant defense pathways that are activated by these phytophagous arthropods, characterized by different feeding habits. Aphids, as well as the few other piercing/sucking insect species studied so far, by producing relatively limited tissue damage, induce defense-signaling pathways (salicylate dependent, jasmonic acid/ethylene dependent) most commonly activated by bacterial, fungal, and viral pathogens (for review see Walling, 2000; Moran and Thompson, 2001). In contrast, spider mites are cell content feeders, causing substantial cellular damage and induce jasmonic acid-dependent wound-response genes, even though salicylate also plays a role in signal transduction (Ozawa et al., 2000; Arimura et al., 2002). However, a shorter time of incubation (12 hr) in a solution containing the regurgitant of a beet armyworm caterpillar *Spodoptera exigua* (Hübner) was sufficient to make a cut stem of a corn seedling attractive to the parasitoids *Cotesia glomerata* (L.) and *Microplitis croceipes* Cresson (Turlings et al., 1993), suggesting that the amount of both leaf damage and elicitor delivered may also play a role.

Even though in our system we have not tested the minimum time of incubation of an uninfested plant, placed in a solution vacated by an infested plant, necessary to make it attractive to female parasitoids, it is reasonable to speculate that this may be related to aphid density and, in turn, to the amount of elicitor(s) released into the medium. Previous research has demonstrated an aphid

density–feeding duration interaction (Powell et al., 1998, Guerrieri et al. 1999) and the use of rhizopshere studies could allow us to determine how much of this interaction can be explained simply by the quantity/quality of an elicitor entering the plant.

The process involved in the exchange of root exudates between plants in the broad bean–*A. pisum*–*A. ervi* system requires further investigation. In particular, it would be interesting to verify for how long the conditioned plant remains attractive; it is already known that an infested plant from which aphids have been removed retains its level of attractiveness to *A. ervi* for 24–48 hr, after which it loses its attractiveness (Du et al. 1997, Guerrieri, unpublished data). It also would be useful to determine if root exudates are herbivore-specific: it has been demonstrated that volatiles released from a broad bean plant infested by the nonhost *Aphis fabae* (Scopoli) were significantly less attractive to *A. ervi* than those from plants infested by the host *A. pisum* (Du et al., 1996; Guerrieri et al., 1999). This could also help in assessing whether the elicitors circulating throughout the plant are intact components of host saliva or are synthesized de novo by the plant in response to host saliva, or if they derive from enzymatic activities present in the aphid watery saliva (Miles, 1999; Walling, 2000). Finally, but equally important, it would be useful to determine if root communication can trigger the activation of plant direct defense pathways against herbivores. However, it will not be easy to unravel the molecular bases of these plant–plant communication mechanisms due to the paucity of information available on root signals implicated in this process.

The results presented in this study may have some interesting ecological implications. Parasitoids are known to use mainly chemical stimuli to locate their victims. In the first steps of host location, as defined by Vinson (1985), the plant can play a key role by producing herbivore-induced volatiles that couple a high reliability (i.e., the presence of the host) to a high detectability (plant volatiles are quantitatively more abundant than host volatiles) (Vet and Dicke, 1992). When uninfested plants are conditioned to emit herbivore-induced synomones by contact with the root exudates of neighboring infested plants, they all lose their individuality and behave in concert. This may serve to enhance the volatile signal that attracts natural enemies of the infesting herbivore, to the benefit of the infested plant. Then, the net result of plant-to-plant conditioning is the creation of a concentrated "focus of attractiveness," that benefits both the plant community and the natural enemy population.

These purely speculative and intriguing scenarios require considerable research to be substantiated. At this stage, it is important to clarify the mechanistic aspects of insect–plant interaction leading to plant signaling, to understand whether these volatiles are part of the complex active mechanisms of plant resistance or are simply by-products that parasitoids successfully exploit. Only by filling this gap in our knowledge, both at physiological and molecular levels, can we provide meaningful insights for developing more realistic ecological theories.

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SYSTEMIC RELEASE OF HERBIVORE-INDUCED PLANT VOLATILES BY TURNIPS INFESTED BY CONCEALED ROOT-FEEDING LARVAE *Delia radicum* L.

N. NEVEU,[1] J. GRANDGIRARD,[1,*] J. P. NENON,[1] and A. M. CORTESERO,[1]

[1]*Laboratoire d'Ecobiologie des Insectes Parasitoïdes*
Université de Rennes 1
Avenue du Général Leclerc
35042 Rennes cedex, France

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Abstract—When attacked by herbivorous insects, many plants emit volatile compounds that are used as cues by predators and parasitoids foraging for prey or hosts. While such interactions have been demonstrated in several host–plant complexes, in most studies, the herbivores involved are leaf-feeding arthropods. We studied the long-range plant volatiles involved in host location in a system based on a very different interaction since the herbivore is a fly whose larvae feed on the roots of cole plants in the cabbage root fly, *Delia radicum* L. (Diptera: Anthomyiidae). The parasitoid studied is *Trybliographa rapae* Westwood (Hymenoptera: Figitidae), a specialist larval endoparasitoid of *D. radicum*. Using a four-arm olfactometer, the attraction of naive *T. rapae* females toward uninfested and infested turnip plants was investigated. *T. rapae* females were not attracted to volatiles emanating from uninfested plants, whether presented as whole plants, roots, or leaves. In contrast, they were highly attracted to volatiles emitted by roots infested with *D. radicum* larvae, by undamaged parts of infested roots, and by undamaged leaves of infested plants. The production of parasitoid-attracting volatiles appeared to be systemic in this particular tritrophic system. The possible factors triggering this volatile emission were also investigated. Volatiles from leaves of water-stressed plants and artificially damaged plants were not attractive to *T. rapae* females, while volatiles emitted by leaves of artificially damaged plants treated with crushed *D. radicum* larvae were highly attractive. However, *T. rapae* females were not attracted to volatiles emitted by artificially damaged plants treated only with crushed salivary glands from *D. radicum* larvae. These results demonstrate the systemic production of herbivore-induced volatiles in this host-plant complex. Although the emission of parasitoid attracting volatiles is induced by factors present in the herbivorous host, their exact origin remains

*To whom correspondence should be addressed. E-mail: Julie.Grandgirard@univ-rennes1.fr

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unclear. The probable nature of the volatiles involved and the possible origin of the elicitor of volatiles release are discussed.

Key Words—*Trybliographa rapae*, *Delia radicum*, tritrophic interactions, host location, herbivore-induced volatiles, systemic release, root-feeders, olfactometer.

INTRODUCTION

When attacked by herbivorous arthropods, at least 23 plant species release volatiles that attract natural enemies of these herbivores (reviewed in Takabayashi and Dicke, 1996; Dicke, 1999b; Sabelis et al., 1999). Herbivore-induced plant volatiles are easily detectable and very reliable cues for foraging natural enemies, since they are produced in large amounts by plants, but only when the plants are under herbivore attack (Dicke, 1999a). Consequently, they are frequently described as the major chemical cues used by foraging parasitoids and predators in long-range host or prey location (Dicke and Sabelis, 1988; Dicke et al., 1990; Turlings et al., 1990; Vinson, 1991; Tumlinson et al., 1992, 1999; Vet and Dicke, 1992; Geervliet et al., 1994; Drukker et al., 1995; van Alphen and Jervis, 1996; Guerrieri et al., 1999; Pickett et al., 1999). In several cases, the attractive volatiles were released not only by the infested parts of plants, but also systemically by uninfested parts of infested plants, which probably further increases the detectability of the signal (Dicke et al., 1990, 1993; Turlings and Tumlinson, 1992; Potting et al., 1995; Röse et al., 1996; Cortesero et al., 1997; Guerrieri et al., 1999). In addition, a few studies have shown that uninfested plants treated with herbivore regurgitant release attractive volatiles, thereby demonstrating that the elicitor of attractive volatile release can originate from herbivores (Turlings et al., 1993; Mattiacci et al., 1994; Alborn et al., 1997). While the release of volatiles attracting parasitoids and predators appears rather widespread in the plant kingdom, in most studies, the herbivores studied to date have been leaf-feeding arthropods (Couty et al., 1999; Dicke, 1999b; Sabelis et al., 1999).

We studied the long-range plant volatiles involved in host location in a system based on a very different interaction, since the herbivore is a fly—the cabbage root fly, *Delia radicum* L. (Diptera: Anthomyiidae)—whose larvae feed on the roots of many cole plants. The parasitoid studied, *Trybliographa rapae* Westwood (Hymenoptera: Figitidae), is a specialist larval parasitoid of a few fly species belonging to the genus *Delia*. In particular, it attacks *D. radicum* L., *D. floralis* Fall., and *D. platura* Meig. (Wishart and Monteith, 1954; Wishart, 1957; Wishart et al., 1957). It is a koinobiont endoparasitoid that can develop on the three developmental stages of *D. radicum* (Jones, 1986; Tamer, 1994). All *T. rapae* hosts feed by digging galleries in the roots of their host plants (Jones, 1986). *T. rapae* females use three types of behaviors to locate host larvae buried in the plant tissues: antennal searching, ovipositor probing, and vibrotaxis (Vet and van Alphen, 1985). If larvae are located beneath the skin of the root, females

probe through the plant tissues with their ovipositor, but if larvae are concealed in the middle of the root, females slip through the galleries to reach them (Jones, 1986).

Little is known about the role of plant volatiles in host habitat location and host location of *T. rapae*. Vet (1985) and Jones (1986) have demonstrated that *T. rapae* females are attracted from a distance by volatiles emanating from *Brassica* roots and can distinguish volatiles from uninfested over infested and/or naturally decaying roots.

Because host larvae are concealed in galleries located inside buried cabbage roots, long-distance host location may be especially difficult in this parasitoid, in particular, no visual signals are available to detect damaged plants at a distance. In such a context, volatile cues may be of special importance, but they may be difficult to detect if released only by the buried root.

In the present work, we determined the attractiveness of different parts of uninfested and infested turnip plants for *T. rapae* females using a four-arm olfactometer. The possible factors triggering the emission of female-attractive volatiles were also investigated.

METHODS AND MATERIALS

Insects

The *D. radicum* culture originated from a colony maintained by the Institut National de la Recherche Agronomique (INRA) in Le Rheu, Brittany, in France.

T. rapae originated from *D. radicum* pupae collected from field Crucifera in Brittany in 1994. The strain has been refreshed yearly. The parasitoid was reared in a climate controlled room ($20 \pm 1^{\circ}$C, $60 \pm 10\%$ relative humidity, 16L:8D), on larvae of *D. radicum* fed on roots of turnip (*Brassica campestris* L.) or swede (*B. napus* L.) according to the rearing technique described in Neveu et al. (1996). After emerging from pupae, 5–10 females were kept with the same number of males in a plastic Petridish (8 cm diam. 2.5 cm high) that contained acacia honey and damp cotton wool. Food and water were regularly replenished.

All experiments were conducted with 3–6-day-old mated females. The females had no previous experience with plant or larval volatiles and had no oviposition experience. At least 1 hr before conducting the olfactometric tests, females were introduced individually in to plastic Petridishes placed in the olfactometer room.

Plants

Turnip plants were obtained from commercial roots (undetermined variety) that weighted approximately 100 g. They were placed in a climate controlled room ($20 \pm 1^{\circ}$C, $60 \pm 10\%$ RH., 16L:8D) where whole plants were grown in

350-ml plastic pots containing damp sand. Plants were watered every 2–3 days and kept for 1–2 weeks until they developed 3–10 new leaves.

Olfactometer Setup

Responses of *T. rapae* females to volatile chemicals emitted by different odor sources were measured in a four-arm olfactometer (Pettersson, 1970; Vet et al., 1983) with some modifications. Reconstituted compressed air (80% nitrogen and 20% oxygen) was circulated through a water bottle before entering the exposure chamber through the four arms. Air left the chamber through a hole in the chamber floor that was covered with a fine mesh to prevent insects from escaping the chamber. The chamber was constructed of Plexiglas. Its internal dimensions were 10 mm in height and 93 mm at its narrowest width across. The top Plexiglas plate had a central hole (12 mm diam.) closed with a plug.

Airflow in each of the four arms was adjusted with a flowmeter (Shorate 150 with glass float, Fisher-Rosemount) to 200 ml/min, thereby creating four distinct fields that were equal in size in the exposure chamber. These fields were visualized with a black background and smoke (chemical reaction of NH_4OH and HCl). The sharpest edges were produced with a flow rate of 200 ml/min through each arm. Therefore, experiments were conducted with an airflow of 200 ml/min. After circulating through the olfactometer, the air was released outside the experimental room.

To deliver the odor to one of the four fields of the olfactometer, the corresponding arm was connected with silicon tubing to a plastic box ($24 \times 18 \times 10$ cm) containing the odor source and tightly closed with adhesive plastic tape. Empty plastic boxes were connected to the control odorless fields (pure air). A circular neon light source underneath the olfactometer provided even illumination of 400 lux in the exposure chamber. The olfactometer setup was placed in a temperature controlled room ($19 \pm 2^{\circ}$C). Preliminary tests with 72 females showed that in pure air, female behavior was similar in the four fields of the olfactometer.

Bioassay Procedure

Individual female parasitoids were introduced through the top hole of the exposure chamber and placed on the fine mesh. Observations started when the female left the center and lasted for 2 min. If the female left the exposure chamber through one of the arms and entered the silicon tubing or the plastic box containing the odor source for more than 10 sec (a period after which females were never observed returning to the exposure chamber in preliminary experiments), the test was terminated and the remaining experimental time was counted as being spent in that field. Every 10–15 observations, the position of the odor field(s) was changed and the exposure chamber was washed with 95% alcohol and an antistatic chemical

(Altunet). Between odor trials, the chamber and the plastic boxes were washed with a neutral detergent (RBS 25) and rinsed with water. We measured the time spent in each field. Parasitoids were discarded if they: needed more than 5 min to leave the center; stayed only in the half of the odor field contiguous to the center; did not move for more than 60 sec; or returned for more 30 sec to the center. Insect paths in the exposure chamber were recorded with a video camera placed on top of the olfactometer.

Odor Sources

Each odor source consisted of one plant (part or whole) per plastic box. Plants were either uninfested or infested. Infested plants were obtained by depositing approximately 100 *D. radicum* eggs, 0–24 hr old with a small paintbrush. They were tested in the olfactometer 5–7 days after the infestation occurred. In all cases, only roots were infested, as *D. radicum* larvae are strictly root feeders.

When leaf volatiles were tested in the olfactometer, a special device was used to isolate the soil, the root, and the plant collar from the leaves over which the air was blown. This device consisted of a plastic cover with a 1-cm-diam. hole in the middle placed on the pot at the time of planting. Each cover was tightly closed with adhesive tape around the pot, and the hole was elongated with a vinyl sleeve (at least 3 cm in height) and Teflon tape, which covered the plant collar.

Response of T. rapae Females toward Uninfested Plants. The following odor sources were tested: uninfested whole plant (WP-UNINF); uninfested root (Rt-UNINF); and leaves from an uninfested plant (Lf-UNINF). In all cases, plants were never exposed to any larval damage.

Response of T. rapae Females toward Infested Plants. The following odor sources were tested:

1. infested root (Rt-INF): The eggs used to infest the root were deposited on the internal face of two halves of a root, placed on damp sand in a glass dish (15 cm diam.).

   For the following 2 odor sources, a root was cut in half. One half was used for UndRt-INF and the other for UndRt-UNINF.
2. Undamaged part of an infested root (UndRt-INF): One half of a root was infested. Just before the olfactometric tests, the damaged part, i.e., the part that contained the larvae, their tunnels, and any damaged tissue, was removed and the remaining part was tested.
3. Undamaged part of an uninfested root (UndRt-UNINF): The other half of the root was laid on damp sand and was left uninfested. Approximately the same part of the root as above was removed.
4. Leaves of an infested plant (Lf-INF): To infest the plant, the eggs were deposited on the top of the root.

Origin of Attractive Volatiles. The following odor sources were tested:

1. Leaves of water stressed plants (Lf-STRESS): Plants were only watered at the time of planting. At the time of the olfactometric tests, leaves were slightly withered.
2. Leaves of artificially damaged plant (Lf-ARTIF): Ten superficial cuts were made with a scalpel (about 1 cm in length, 0.5 cm in depth) at the plant's collar. To mimic feeding behavior of the root-feeder larvae, this treatment was repeated each day for 4 consecutive days. Olfactometric tests were conducted 4–6 hr after the last cuts were performed.
3. Leaves of artificially damaged plants treated with crushed larvae (Lf-ARTIF + CL): Twenty-five 5- to 7-day-old larvae were washed in distilled water, dried on filter paper, and crushed with a glass rod in a drop of water onto a slide. The liquid obtained was applied with a paintbrush on the cuts of an artificially damaged plant obtained as described above. New crushed larvae were applied to fresh artificial damage each day for 4 consecutive days.
4. Leaves of artificially damaged plants treated with larval salivary glands (Lf-ARTIF + SG): Twenty-five 5- to 7-day-old *D. radicum* larvae were dissected and their salivary glands were extracted with precision forceps and placed in a Ringer solution. Each pair of glands was torn apart, crushed with a needle, and then applied with a paintbrush on the cuts of an artificially damaged plant obtained as described above. New glands were applied to fresh artificial damage each day for 4 consecutive days.

Data Analysis

Homogeneity of times spent in fields of the olfactometer was analyzed by using ANOVA. Pairwise comparisons of time spent in each of the four fields were made by using Scheffe tests.

RESULTS

Response of T. rapae Females toward Uninfested Plants. Naive *T. rapae* females were not attracted to volatiles emanating from an uninfested turnip plant. Volatiles from either whole plants (Figure 1A) or leaves (Figure 1C) did not increase the time spent in the odor field compared to pure air ($F = 0.47$, $df = 3$, $P > 0.05$ vs. $F = 2.28$, $df = 3$, $P > 0.05$). Females preferred the pure air fields to the one odorized with an uninfested root, indicating possible avoidance ($F = 7.76$, $df = 3$, $P < 0.001$) (Figure 1B).

Response of T. rapae Females toward Infested Plants. Volatiles emanating from a root infested with *D. radicum* larvae were attractive to females (Figure 2A) ($F = 19.55$, $df = 3$, $P < 0.001$). Females also preferred volatiles from the

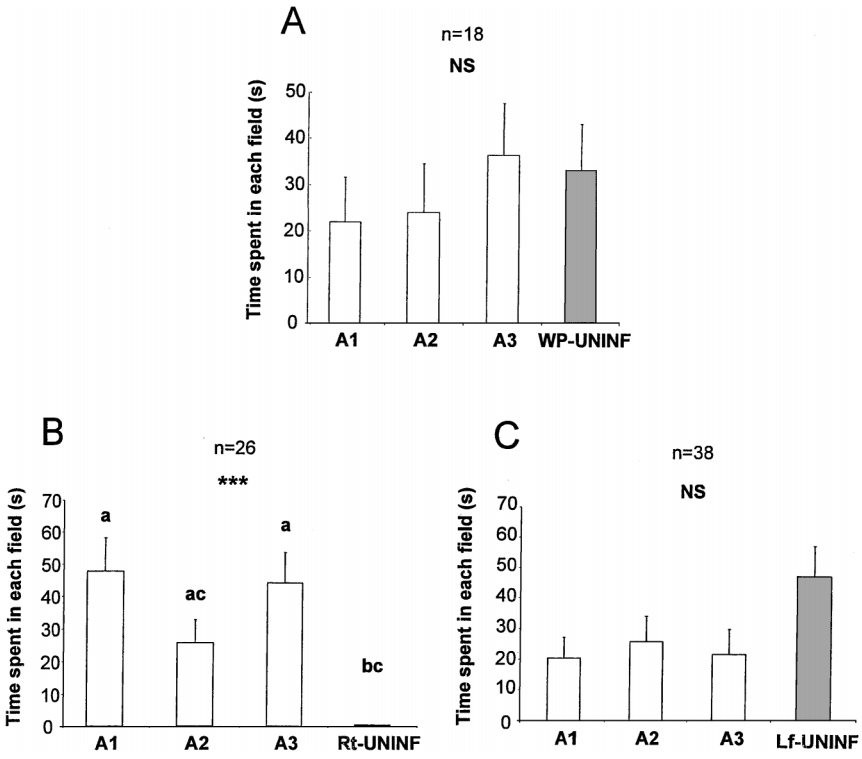


FIG. 1. Mean time spent (±SE) in each field of a four-arm olfactometer by inexperienced *T. rapae* females exposed to voltatiles from uninfested turnips. One field tested the volatiles from (A): a whole turnip (WP-UNINF), (B) a root (Rt-UNINF), or (C) leaves (Lf-UNINF). The other three fields tested pure air (A1, A2, A3). Means with different letters are significantly different (ANOVA followed by Scheffé test). NS: no significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

undamaged part of an infested root to volatiles from the undamaged part of an uninfested one (Figure 2B) ($F = 25.26$, $df = 3$, $P < 0.001$). Furthermore, they preferred volatiles from undamaged leaves of root-infested plant to volatiles from undamaged leaves of an uninfested plant (Figure 2C) ($F = 42.19$, $df = 3$, $P < 0.001$).

Origin of Attractive Volatiles. As leaves of root-infested plants were highly attractive, the factors responsible for the emission of attractive volatiles were investigated. Volatiles emanating from leaves of a water-stressed plant were not attractive (Figure 3A) ($F = 0.49$, $df = 3$, $P > 0.05$). Volatiles emanating from leaves of an artificially damaged plant were not attractive either; the females tended to prefer pure air (Figure 3B) ($F = 3.44$, 3 $df = 3$, $P < 0.05$). However, females preferred

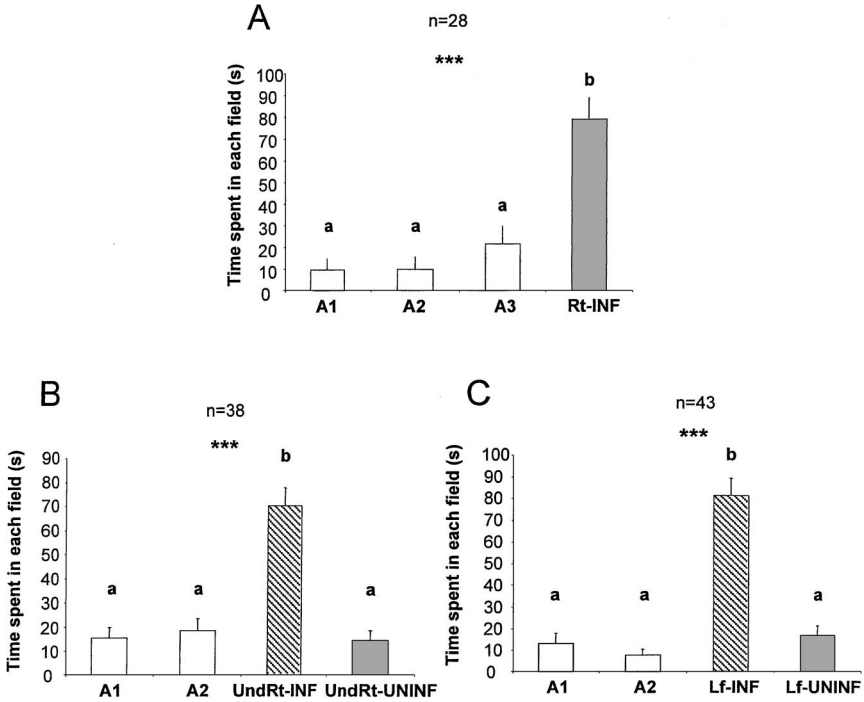


FIG. 2. Mean time spent (±SE) in each field of a four-arm olfactometer by inexperienced *T. rapae* females exposed to volatiles from infested turnips. (A) One field tested the volatiles from an infested root (Rt-INF), and the other three fields tested pure air (A1, A2, A3). (B and C) One field tested the volatiles from either a root (Rt-INF)(B) or leaves (Lf-INF)(C) of an infested turnip, and another field tested the volatiles from either a root (Rt-UNINF)(B) or leaves (Lf-UNINF)(C) of an uninfested turnip, while the other two field tested pure air (A1, A2). Means with different letters are significantly different (ANOVA followed by Scheffé test). NS: no significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

volatiles from artificially damaged plants treated with crushed larvae to volatiles from artificially damaged plants only or to pure air ($F = 26.40$, $df = 3$, $P < 0.001$) (Figure 3C). When an artificially damaged plant was treated with larval salivary glands, females were not attracted to volatiles from its leaves, when tested against volatiles from leaves of an artificially damaged plant and pure air (Figure 3D) ($F = 2.73$, $df = 3$, $P > 0.05$).

DISCUSSION

Response to Plant Volatiles. Our study demonstrated that *T. rapae* females are not attracted to volatiles emanating from uninfested turnips whether presented

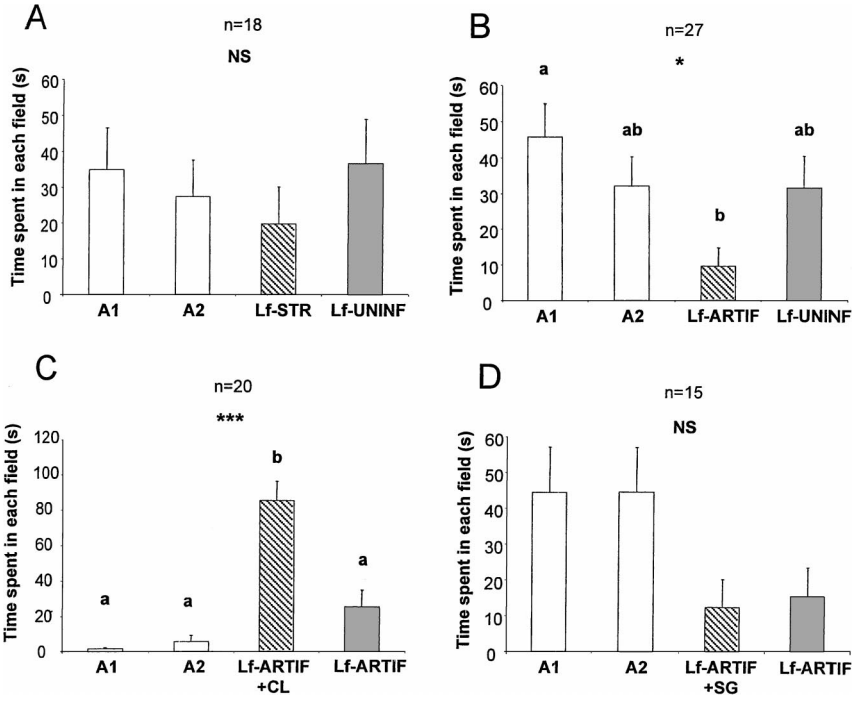


FIG. 3. Mean time spent (±SE) in each field of a four-arm olfactometer by inexperienced *T. rapae* females exposed to leafs odor of uninfested turnips submitted to different treatments. (A and B) One field tested volatiles from either a water-stressed turnip (Lf-STR)(A) or an artificially damaged turnip (Lf-ARTIF) (B), and another field tested volatiles from an uninfested plant (Lf-UNINF), while the other two fields conducted pure air (A1, A2). (C and D) One field tested the volatiles from an artificially damaged turnip treated either with crushed larvae (Lf-ARTIF+CL)(C) or salivory glands (Lf-ARTIF+SG(D), and another field tested the door of artificially damaged turnip (Lf-ARTIF); the other two fields tested pure air (A1, A2). Means with different letters are significantly different (ANOVA followed by Scheffe test). NS: no significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

as whole plants, roots, or leaves. This lack of response toward uninfested plants has been observed in other parasitoid species (Turlings et al., 1990; Guerrieri et al., 1993; Potting et al., 1995; Finidori-Logli et al., 1996; Du et al., 1996; Steidle and Schöller, 1997) and may be attributed to the low reliability of this signal to inform the parasitoid of host presence (Vet et al., 1991; Vet and Dicke, 1992).

In contrast, our results demonstrated that volatiles emanating from infested turnip roots were attractive to *T. rapae* females that are able to distinguish uninfested turnip roots from infested turnip roots.

We showed that volatiles emanating from the undamaged part of an infested root were more attractive than volatiles from an uninfested root. This demonstrates that the emission of attractive volatiles is not restricted to the infested part of the root. Moreover, attractive volatiles are emitted not only by the root itself but also by undamaged leaves of a damaged plant. Indeed, *T. rapae* females preferred volatiles from the leaves of an infested turnip to volatiles from uninfested turnip leaves. Therefore, it can be concluded that volatiles attracting *T. rapae* females are emitted systemically by plants infested by *D. radicum* root-feeding larvae. Systemic release of volatiles under herbivore attack has been demonstrated in four other plant species: lima bean (*Phaseolus lunatus*) (Dicke et al., 1990), corn (*Zea mays*) (Turlings and Tumlinson, 1992; Potting et al., 1995), cotton (*Gossypium hirsutum*) (Röse et al., 1996, Cortesero et al., 1997), and broad bean (*Vicia faba*) (Guerrieri et al., 1999). Our study demonstrates for the first time, That most herbivores attacking these plant species feed on aerial parts of plants; the emission of parasitoid-attracting volatiles from leaves of plants receive herbivore attack at the root level. Systemic release of plant volatiles is advantageous for *T. rapae* foraging females because *D. radicum* are concealed in the roots, whereas parasitoids forage for hosts in flight.

Turnips become attractive to *T. rapae* females only after being infested by *D. radicum* larvae. The volatiles involved in this attraction could be isothiocyanates, the volatiles specific to the genus *Brassica*. In this genus, the development of the turnip root fly *D. floralis*, a species close to *D. radicum*, induces chemical modifications in the plants attacked (Birch et al., 1990, 1992; Hopkins et al., 1993, 1995). In particular, larval damage to roots induces a modification in leaf glucosinolate composition: the concentration in aliphatic glucosinolates increases and the concentration in indole-based glucosinolates decreases (Birch et al., 1992). Glucosinolates are nonvolatile precursors of volatile isothiocyanates. Based on these results, it is plausible that *T. rapae* females modify leaf isothiocyanates and that this modification is induced by *Delia* spp. larval attacks on the roots. Additional chemical analysis is necessary to confirm this hypothesis, however.

Origin of Attractive Volatiles. The damage inflicted by *D. radicum* larvae on turnip roots usually generates water stress. This stress could induce the release of volatiles attracting *T. rapae*, as has been shown in lima bean (*Phaseolus lunatus*). Takabayashi et al. (1994) demonstrated that females of the acarian predator *P. persimilis* preferred volatiles emitted by water-stressed plants to those from nonstressed plants. However, in our study, volatiles emanating from leaves of a water-stressed uninfested turnip were not more attractive for *T. rapae* than volatiles from leaves of normally watered uninfested turnips. Therefore, water stress does not appear to induce the release of attractive volatiles.

Another possible factor triggering the emission of volatiles could be mechanical damage caused by *D. radicum* larvae. In many host-plant complexes, volatiles

released by an artificially damaged plant, such as green leaf volatiles, are attractive for parasitoids (Turlings et al., 1990; Whitman and Eller, 1990; McAuslane et al., 1991, Steinberg et al., 1993; Geervliet et al., 1994; Mattiacci et al., 1994; Potting et al., 1999). However, an artificially damaged turnip was not attractive for *T. rapae* females. In most cases, the release of the compounds occurs immediately after the damage and then rapidly decreases (Turlings et al., 1995). In our experiments, the damage was done made each morning for 4 consecutive days, and the olfactometric tests were performed in the afternoon of the fourth day. Consequently, green leaf volatiles were probably released in low amounts during the olfactometric tests.

Finally, the release of volatiles could be due to the contact of plant tissues with one or several compounds originating from *D. radicum* larvae. When an artificially damaged plant was treated with crushed larvae, its leaves very attractive for *T. rapae* females. This result demonstrates that the elicitor of volatiles systemic release originated from the host larvae and that the attractive volatiles emitted are herbivore-induced volatiles.

Herbivore-induced volatiles have been found in other systems involving leaf feeder or leafminer caterpillars (Petitt et al., 1992; Potting et al., 1995; Dutton et al., 2000). In these systems, the elicitor often originated from caterpillar saliva, since parasitoids are attracted by uninfested plants treated with caterpillar regurgitant (Turlings and Tumlinson, 1992; Turlings et al., 1993, Mattiacci et al., 1994; Potting et al., 1995). A few elicitors have been identified (Mattiacci et al., 1995; Alborn et al., 1997; Felton and Eichenseer, 1999). One of them was isolated from the saliva of the beet armyworm *Spodoptera exigua*, and named volicitin [*N*-(17-hydroxylinolenoyl)-L-glutamine] (Alborn et al., 1997). Volicitin is the most active compound of the herbivore saliva and is responsible for the release by maize of volatiles attracting parasitoids of the genus *Cotesia* and *Microplitis croceipes* (Turlings et al., 2000). Recent studies have shown that volicitin and related compounds are present in the saliva of more than 10 different Lepidoptera species (Pohnert et al., 1999; Halitschke et al., 2001; Mori et al., 2001). The second elicitor identified is the enzyme β-glucosidase. This enzyme is present in the regurgitant of *Pieris brassicae* caterpillars and triggers the release of volatiles by Brussels sprouts (Mattiacci et al., 1995). Placed in a solution of this enzyme, a leaf releases volatiles as attractive to *Cotesia glomerata* females as those emitted by an artificially damaged plant treated with caterpillar regurgitant.

Because of the results obtained in these two studies, we hypothesized that the elicitor also would be present in the saliva of *D. radicum*. However, our results showed that attractive volatiles were not released when salivary glands of *D. radicum* larvae were applied to an artificially damaged turnip. In our study, it was not possible to collect oral secretions by squeezing the larvae as was done with *S. exigua* or *P. brassicae* caterpillars (Turlings and Tumlinson, 1992; Mattiacci

et al., 1994). Therefore, we chose to apply dissected crushed salivary glands instead. The digestion of plant tissues is external in dipteran phytophagous larvae (Grassé, 1951), and it is possible that the portion of saliva collected with our method was ineffective or that the amount of saliva was insufficient to induce volatile release.

Another explanation is that the elicitor of volatile release is not present in salivary glands but in another part of the larvae, such as the digestive tract. Indeed, digestive caeca could enclose microorganisms that could induce plant release of attractive volatiles. Effects of microorganisms on volatile compound production has already been demonstrated in several studies (Croft et al., 1993 in Bruin et al., 1995; Doughty et al., 1996). In the *Cruciferae–D. radicum* system, several bacteria are involved in the decay of plant tissues that follows feeding by dipteran larvae (Johnson, 1930; Doane and Chapman, 1964). These bacteria are present in the digestive tract and in adult feces, on eggs and larvae, and inside the puparia of *D. radicum*. They are also present in the digestive tract of the figitid and staphylinid parasitoids of *D. radicum* (Johnson, 1930). The influence of these bacteria on the attraction of *T. rapae* females toward leaves remains to be investigated.

We have shown that infested turnips systemically emit herbivore-induced plant volatiles that attract the parasitoid *T. rapae*. Our next objective is to study the costs and benefits of these volatiles for the plant. As underlined by Turlings and Benrey (1998) and van der Meijden and Klinkhamer (2000), it has long been considered that plants have evolved mechanisms to actively attract natural enemies of their herbivores. If this hypothesis is true, attracting parasitoids should result in increased plant fitness. Until now, only one study has demonstrated increased fitness (seed production) when comparing a plant infested by unparasitized herbivores and one infested by parasitized herbivores (van Loon et al., 2000). However, herbivore-induced plant volatiles can be perceived and used not only by natural enemies of herbivores, but also by all other organisms present in the environment, e.g., herbivores, predators of parasitoids, hyperparasitoids, and neighboring plants, and such organisms could negatively impact plant fitness. For example, it seems that in most systems, many herbivores are attracted rather than repelled by volatiles released by infested plants. Indeed, it has been shown that herbivores can exploit these plant signals to locate their host plants and to get information on the presence of competitors and/or on the defensive state of the plant (Dicke and Vet, 1999; Dicke and van Loon, 2000). Such questions should be addressed in the *T. rapae–Cruciferae–D. radicum* complex to ascertain whether herbivore-induced volatiles are really part of the active defense system of turnip plants.

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Lygus hesperus FEEDING AND SALIVARY GLAND EXTRACTS INDUCE VOLATILE EMISSIONS IN PLANTS[1]

CESAR RODRIGUEZ-SAONA,[2,4] STEVEN J. CRAFTS-BRANDNER,[2,*] LIVY WILLIAMS III,[3] and PAUL W. PARÉ[4]

[2]*USDA-ARS Western Cotton Research Lab.*
Phoenix, Arizona 85040, USA

[3]*USDA-ARS Southern Insect Management Research Unit*
P.O. Box 346, Stoneville, Mississippi 38776, USA

[4]*Texas Tech University, Department of Chemistry and Biochemistry*
Lubbock, Texas 79409, USA

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Abstract—Induction of plant volatiles by leaf-chewing caterpillars is well documented. However, there is much less information about volatile induction by insects with different feeding habits. We studied the induction of plant volatiles by a piercing–sucking insect, the western tarnished plant bug *Lygus hesperus* Knight. Adults of both genders and nymphs of *Lygus* induced the local emission of a blend of volatiles from both cotton and maize. Feeding by *Lygus* also induced the systemic emission of volatiles that was similar but less complex than the blend emitted at the site of feeding. Infestation by mated, mature adult females (>4 days old), but not by nymphs or mature males, caused detectable emission of α-pinene, myrcene, and (*E*)-β-caryophyllene, compounds that are stored in the glands of cotton tissue. This indicated that damage to glands in the petiole and leaf by the female ovipositor, rather than feeding, contributed significantly to the emission of these volatiles. Girdling the plant stem to disrupt phloem transport markedly decreased the movement of ^{14}C-labeled photosynthetic products to the apex of the plant, and this treatment also markedly reduced the amount of systemically induced volatiles caused by *Lygus* feeding. *Lygus* salivary gland extracts were capable of inducing emission of the same volatile blend as measured for plants infested by feeding insects or treated with volicitin, an elicitor isolated from caterpillar regurgitant. The results indicate that

* To whom correspondence should be addressed. E-mail: scraftsbrandner@wcrl.ars.usda.gov

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[5]Current address: Department of Botany, University of Toronto, 25 Willcocks Street, Toronto, Ontario M5S 3B2 Canada.

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L. hesperus is capable of inducing the emission of plant volatiles and that induction is caused by an elicitor that is contained in the insect salivary gland.

Key Words—*Lygus hesperus*, induction, volatiles, systemic signal transport, salivary glands, volicitin.

INTRODUCTION

Herbivore feeding induces plants to emit a blend of volatiles that in turn attracts natural enemies of the herbivores (e.g., Turlings et al., 1995; Dicke, 1999). These herbivore-induced volatiles are emitted not only at the site of herbivore feeding, but also systemically from undamaged portions of the plant (Turlings and Tumlinson, 1992; Röse et al., 1996). This activation and release of volatile organic compounds (VOC's) is considered fundamental in associations involving plants, phytophagous insects, and their natural enemies and is well documented for interactions between plants and leaf chewing caterpillars (Lepidoptera) (see review by Dicke and Vet, 1999). Such leaf-eating larvae can activate plant defense responses by introducing enzymatic or other chemical signals present in the regurgitant of the caterpillar. At least two elicitors, a β-glucosidase identified from *Pieris brassicae* L. gut regurgitant (Mattiacci et al., 1995) and *N*-(17-hydroxylinolenoyl)-L-glutamine (volicitin) isolated from *Spodoptera exigua* Hübner oral secretions (Alborn et al., 1997), have been shown to induce the emission of plant volatiles. Identification of volicitin and other structurally related compounds from several members of the Lepidoptera (Pohnert et al., 1999; Mori et al., 2001; Halitschke et al., 2001), suggests a common interspecies plant–insect signaling mechanism.

Phloem-feeding insects (Homoptera) also induce plant volatiles that attract natural enemies. Aphid-induced emission of plant volatiles can mediate foraging behavior of aphid parasitoids (e.g., Du et al., 1998; Powell et al., 1998; Guerrieri et al., 1999), but to date there are no reports of the mechanism by which aphids activate plants or trigger systemic VOC emissions.

Lygus spp. are pests with feeding traits shared by chewing and sucking insects. As other members of the Heteroptera, *Lygus* bugs have modified piercing-sucking mouthparts (Miles, 1972). The mandibular stylet that repeatedly penetrates the plant tissue causes mechanical damage (Strong, 1970; Tingey and Pillemer, 1977), while the uptake of liquefied plant components defines it as a sucking insect. This uptake of plant components occurs after constituents from the insect's saliva, including digestive enzymes, are injected into the plant tissue (Cohen, 1998; Agustí and Cohen, 2000).

This study was initiated to examine the induction of VOC emissions in plants by the polyphagous herbivore *Lygus hesperus* Knight (Hemiptera: Miridae). Thus far, most studies of VOC activation by herbivore feeding have focused on chewing lepidopterans even though piercing–sucking hemipterans such as *Lygus* spp. can

do significant agricultural damage by feeding on reproductive structures, leaves, stems, and petioles (Wilson et al., 1984). *Lygus hesperus* is found on over 100 plant species (Scott, 1977) and is an important pest in many crops, including legumes, cereals, fruits, vegetables, and various seeds and grains (Tingey and Pillemer, 1977). Here, we addressed the following specific questions: (1) Does *L. hesperus* feeding induce local and systemic emission of volatiles in plants? (2) Is volatile induction affected by *L. hesperus* developmental stage or gender? (3) If induction is systemic, how is the systemic signal transported within the plant? (4) Can emission of plant volatiles be induced by extracts of *L. hesperus* salivary glands?

METHODS AND MATERIALS

Plants and Insects. Four- to 5-week-old cotton (*Gossypium hirsutum* L. cv. Delta Pineland 5415) plants with 4–6 fully expanded true leaves and 10- to 14-day-old maize (*Zea mays* L. var. LG-11) seedlings were used for the experiments. Both plant species are suitable hosts for *L. hesperus* (Scott, 1977).

Cotton plants were grown in insect-free greenhouses as described in detail by Rodriguez-Saona et al. (2001). Maize plants were grown indoors in 16-cm-diam. pots using Pro-Gro potting soil with 8 g Osmocot 14-14-14 fertilizer (Scotts-Sierra Horticulture, Marysville, Ohio, USA) added to the soil at the time of planting. High-pressure sodium and metal halide lamps provided a light intensity of 700 μmol photons/m^2/sec. Room temperature was maintained at $29 \pm 4^{\circ}$C with $40 \pm 10\%$ relative humidity under a 16-hr photoperiod and an 8-hr dark period.

Adults of the western tarnished plant bug, *L. hesperus*, were collected from alfalfa fields (Maricopa, Arizona) and held in 30- $\times$ 30- $\times$ 30-cm polypropylene cages (Megaview Science Education Services Co., Ltd., Taichung, Taiwan) placed in environmental chambers maintained at $25 \pm 2^{\circ}$C and $65 \pm 10\%$ relative humidity under a 14L:10D photoperiod. *Lygus* nymphs and adults were maintained on a diet that consisted of fresh green beans and carrots, supplemented with pink bollworm eggs (Beards and Leigh, 1960) or on artificial diet (Debolt, 1982). *Spodoptera exigua* larvae were reared on an alfalfa-based diet (Henneberry and Kishaba, 1966) and maintained at $28 \pm 2^{\circ}$C and 50% relative humidity under a 14L:10D photoperiod. Both insect colonies were maintained at the Western Cotton Research Laboratory, Phoenix, Arizona.

Collection and Analysis of Plant Volatiles. For all experiments, volatiles were collected from plants maintained in the greenhouse (cotton) or the plant growth room (maize). Cotton volatiles were collected using a push–pull volatile collection apparatus constructed after Heath and Manukian (1994) [see Rodriguez-Saona et al. (2001), for details]. Charcoal-purified air entered from the top of a glass chamber and passed over a plant at a rate of 5 liters/min. Plant volatiles were collected by pulling 20% of the air inside of the chamber (1 liter/min rate) through

a Super-Q adsorbent (Alltech, Deerfield, Illinois, USA) filter trap. Volatiles from maize seedlings were collected using a 60-cm-high × 14-cm-diam. glass cylinder placed over the aerial portion of plants. Air was passed over the plants at 3 liters/min and was pulled through filter traps at 1 liter/min. The remainder of the air was vented out the bottom of the system through the opening, loosely sealed with cotton, around the stem of the plant.

A system with four independent chambers allowed for simultaneous collection of volatiles from four plants. Cotton volatiles were extracted from the collection filters and analyzed by gas chromatography (GC) as described by Rodriguez-Saona et al. (2001). Volatiles from maize seedlings were extracted from traps with 150 μl methylene chloride. An internal standard (*n*-nonyl acetate 40 ng/μl) in 5 μl of methylene chloride was added. Aliquots 1-μl of the extract were injected onto a capillary GC (Hewlett-Packard 6890) with a 15-m × 0.25-mm-ID fused silica column and a 0.1-μm-thick bonded methylsilicone. Injections were made in a splitless mode for 0.3 min with an injector temperature of 230°C and the detector temperature of 250°C. The column was held at 40°C for 0.5 min, increased 12°C/min to 180°C, and then increased to 40°C/min to 220°C and held for 2 min. Helium was used as a carrier gas at a linear flow velocity of 35 cm/sec. For each sample, volatiles were quantified based on comparison of their peak areas with that of the internal standard. Compounds were identified by GC–mass spectroscopy (GC-MS) as described by Rodriguez-Saona et al. (2001); spectral data were compared with synthetic standards from commercial sources and spectra from the National Institute of Standards and Technology (NIST, 1995) database.

Volatile Emission from Plants Infested with Lygus. Thirty to 40 adult bugs (mixed sexes) were collected from the colony and placed on a plant 18 hr prior to collecting volatiles in order to allow the insects to become conditioned to the new diet. Preliminary observations showed that this insect density was sufficient to cause significant induction of volatile emission and also allowed for continued insect growth and development. Volatile collection was initiated at 10:00 hr. Volatiles from cotton plants were collected continuously for 8 hr, while maize volatiles were collected for 6 hr/day during two consecutive days. Controls were included to test for volatile emission from plants with no insects or from chambers that contained 30–40 adult bugs with a source of water but no plants. In some cases, insects were removed from the plants prior to placing the plants in the collection chambers. Each treatment was replicated at least four times.

To determine the effects of developmental stage and gender on induction of plant volatiles, emitted volatiles were measured after placing nymphs (fourth or fifth instars) or sexually mature adult (>4 days old) females or males of *L. hesperus* on cotton plants. Thirty to 40 individuals from each group were placed on the plants for 18 hr prior to initiating volatile collection at 10:00 hr on the following morning. Samples were collected continuously for 8 hr. Each treatment was replicated at least four times. In addition, the total number of eggs oviposited

on each plant by adult females was recorded. Gender differences in induction of VOC emissions were also tested for maize plants.

Systemic Induction of Volatiles by Lygus Feeding. To determine if feeding by *Lygus* triggers a systemic response in cotton, 30–40 adult *L. hesperus* (mixed sexes) were restricted to two lower leaves of a cotton plant while volatiles were collected from the portion of the plant above the infested leaves. Volatiles were collected as previously described with the difference that only the upper portion (undamaged leaves) of plants was enclosed inside the collection chamber, while the lower two leaves where the insects were feeding remained outside (see Röse et al., 1996, for details). *Lygus* bugs were contained on the lower leaves by using fine-mesh nylon bags sealed at the opening with Velcro. Control plants had two lower leaves enclosed by empty bags. Volatiles were collected for a 32-hr period by collecting for 8 hr/day beginning at 10:00 hr on each of four consecutive days. Treatments were replicated at least four times.

To test if the systemic induction of volatile emissions in cotton is mediated by chemical signals transported via the phloem, phloem transport was disrupted between the site of herbivory and the portion of the plant from which volatiles were collected. The stems of cotton plants were girdled by using a bare constantan wire (approximately 0.7 mm diam.) that was heated electrically with a variable AC voltage controller set at 5% of maximum power (Bruening and Egli, 1999). The wire was wrapped around the stem and heated for 5 sec, removed, then wrapped around the stem in the opposite direction and heated again for 5 sec. This treatment caused a large burned circle around the stem that cut deep into the tissue such that, upon dissection, no living tissue was visible. The girdled plants were secured to stakes and left overnight without further treatment to ensure that xylem transport was not disrupted; if the leaves remained turgid the following day, the plant was used for experimentation. All of the phloem-severed plants used for experiments remained turgid during the following week in which plants were visually inspected.

Thirty to 40 sexually mature adult (mixed sexes) *L. hesperus* or four third-instar *S. exigua* were placed on two leaves below the girdled area or on comparable leaves of nongirdled control plants. Volatiles were collected from insect-free leaves above the girdled site as previously described for systemic induction experiments. Volatile emission was also measured for control plants that were girdled and free from insects. Treatments were replicated at least five times.

To test the effectiveness of the girdling treatment, we measured the translocation of ^{14}C from leaves below the girdle to apical portions of the plant. Photosynthetically fixed carbon is transported predominantly in the phloem tissue (Buchanan et al., 2000). A large leaf located below the stem girdle but not used for insect feeding was labeled with $^{14}CO_2$. A comparable leaf for nongirdled controls was also labeled. An airtight bag was placed over the leaf and tightly secured around the petiole with wire. Five hundred microliters of gaseous $^{14}CO_2$ (0.025 μCi/μl)

liberated from acidified [^{14}C] $NaHCO_3$ was injected into the bag. After 3 days, tissue samples were taken from the labeled leaf, from the apex of the plant (small flower buds and very small leaves <1 cm long), and from small leaves (<8 cm long) from the top of the plant. Tissue samples were also collected from plants that were not labeled with $^{14}CO_2$. Immediately after sampling, a small amount of plant tissue was powdered in liquid N_2 and homogenized in 750 μl of 0.5 M HCl. The entire sample was transferred into scintillation vials, mixed with 5 ml of scintillation cocktail and counted. Treatments were replicated at least five times.

Dissection of Lygus Salivary Glands. Adult *L. hesperus* were caged on maize Pioneer var. 3223, for 2–3 days. Cages were held in an environmental chamber maintained at $27 \pm 1^{\circ}$C and $70 \pm 10\%$ relative humidity under a 14L:10D photoperiod. Prior to dissection, insects were aspirated from cages and held at 4°C for ca. 30 min. *Lygus* were pinned in a paraffin-filled Petri dish and submerged in cold (4°C) phosphate-buffered saline (PBS), pH 7.4. Salivary glands were then removed intact and rinsed in cold distilled water.

During dissection of *Lygus* adults it was noted that female salivary glands appeared to be larger than those of males. To determine differences in size between salivary glands of male and female *L. hesperus*, salivary glands were dissected from living insects as described above. Only one gland was measured from each insect. Glands were submerged in a small drop of PBS and measured to the nearest 0.03 mm using a dissecting scope with ocular micrometer at 45× magnification. Ten measurements were made on each gland: maximum lengths of the primary lobe, accessory lobe, and accessory gland; diameters of the primary lobe and accessory lobe at three equidistant locations along the length of each lobe; and maximum diameter of the accessory gland (see Baptist, 1941).

The volume of each salivary gland was estimated by treating the gland as a cylindrical solid, i.e., volume = (length)π[(diameter)/2]^2. Separate calculations were made for the primary lobe, accessory lobe, and accessory gland. For the primary lobe and accessory lobes, we used the mean of the three diameter measurements. Volume estimates for the primary lobe, accessory lobe, and accessory gland were then summed to give the absolute estimate of volume (cubic millimeters) for each salivary gland.

Induction of Maize Volatiles by Lygus Salivary Gland Extracts. Extracts of *Lygus* salivary glands dissected from living females were used to test for activity in inducing plant volatile emission from maize seedlings. One hundred glands were placed together in a 1-ml cone-bottom glass vial with 10 μl of distilled water and held at –80°C prior to the preparation of extracts. Each 100-gland sample was extracted in 50 μl of distilled water by sonicating the samples for 15 min. After centrifuging for 5 min, the supernatant was transferred to a new vial and used for induction experiments. Ten-day-old maize seedlings were cut at the stem close to the soil and incubated for 12 hr at room temperature in a laboratory with no supplemental light starting at 21:00 hr in 1-ml shell vials containing either 10 μl

of *Lygus* salivary gland extract or 10 μl of 30 μM synthetic volicitin, plus an additional 490 μl of a 50 mM sodium phosphate buffer, pH 8.0. Control plants were incubated in 10 μl of distilled water plus 490 μl of buffer solution. The following morning, seedlings were removed from the vials, the severed ends were wrapped in wet cotton, and the seedlings were placed in 37-cm-long $\times$ 4-cm-diam. closed glass cylinders. Air was passed over the plant at a rate of 600 ml/min and was pulled by vacuum through a filter trap attached at the opposite side of the chamber. Volatiles from 12 seedlings were collected simultaneously in parallel glass chambers in the plant growth room. Volatiles were collected for 6 hr (10:00–16:00 hr) and analyzed as previously described. Each treatment was replicated at least five times.

Voucher Specimens. Voucher specimens of *L. hesperus* are deposited in the USDA-ARS Systematic Entomology Laboratory, Beltsville, Maryland, USA.

Statistical Analysis. Differences among treatments were analyzed by using a completely randomized one-way ANOVA (SigmaStat, San Rafael, California, USA). If significant differences were detected, a pairwise multiple comparison procedure (Tukey test) was conducted. Differences between two treatments were analyzed using two-tailed student *t* tests (SigmaStat). The number of eggs oviposited per cotton plant was regressed versus the amount of constitutive volatiles emitted.

RESULTS

Volatile Emission from Plants Infested with Lygus. Lygus infestation of both cotton and maize induced the emission of several plant VOC's (*t* tests; $P < 0.05$; Figure 1). Infestation of cotton and maize plants increased volatile emissions by 12- and 5-fold, respectively, as compared to insect-free controls. For cotton, the herbivore-induced volatiles included (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, indole, (*E*)-β-farnesene, (*E*,*E*)-α-farnesene, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Figure 1). These compounds were induced by infestation with nymphs and adult males and females (data not shown), and many of the compounds were detected even after removing the insects from the plants prior to collecting volatiles (Figure 1). These same inducible volatiles are emitted from cotton plants infested by chewing caterpillars (Paré and Tumlinson, 1997a,b). In addition, cotton plants infested with a mixture of mature males and ovipositing females also emitted large amounts of α-pinene, β-pinene, myrcene, limonene, and (*E*)-β-caryophyllene. These constitutive terpenes are stored in the glands of cotton tissue (Elzen et al., 1985) and are emitted when the glands are mechanically ruptured (Paré and Tumlinson, 1997a,b). There was no detectable emission of the constitutive volatiles from nymph-infested plants (data not shown).

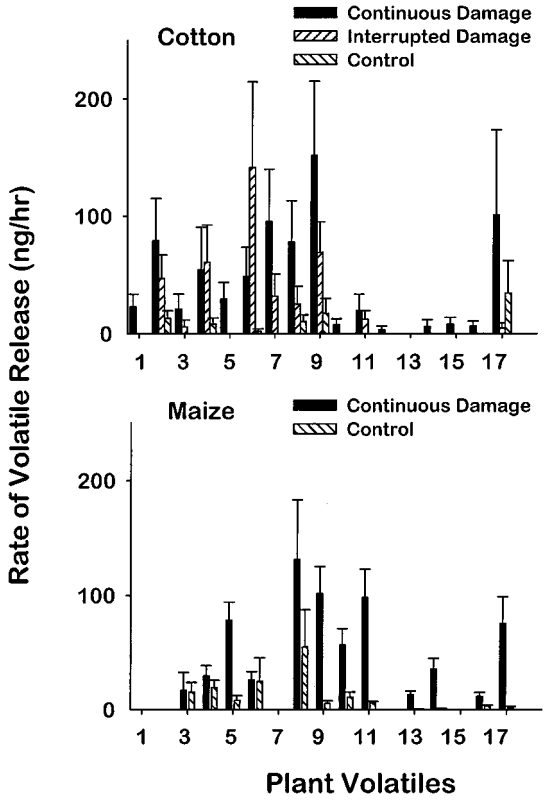


FIG. 1. Volatiles collected from the aerial portions of cotton or maize plants: (1) infested continuously with adult *Lygus hesperus*; (2) from which insects were removed from the plants prior to volatile collection; or (3) maintained free from insects. **1** = (*E*)-2-hexanal; **2** = α-pinene; **3** = β-pinene; **4** = myrcene; **5** = (*Z*)-3-hexenyl acetate; **6** = limonene; **7** = (*E*)-β-ocimene; **8** = linalool; **9** = (*E*)-4,8-dimethyl-1,3,7,-nonatriene; **10** = indole; **11** = (*E*)-β-caryophyllene; **12** = α-humulene; **13** = α-*trans*-bergamotene; **14** = (*E*)-β-farnesene; **15** = (*E*,*E*)-α-farnesene; **16** = nerolidol; **17** = (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Volatiles were collected continuously for 8 hr (cotton) or 6 hr/day over two consecutive days (maize), starting at 10:00 hr. Bars represent the mean + 1 SE; $N \geq 4$. Total cotton volatile emissions (mean $\pm$ 1 SE): continuous damage = 1010.9 ± 399.9; interrupted damage = 397.2 ± 197.6; control = 85.07 ± 40.8. Total maize volatile emissions: continuous damage = 981 ± 190.8; control = 201.4 ± 23.0.

When studied in more detail, emission of constitutive volatiles was significantly higher when mated females were feeding on the plants as compared to males of the same age (data not shown; $t = 2.75$; $df = 12$; $P = 0.017$). Mated females laid an average of 143 ± 16 (mean $\pm$ SE) eggs per plant, and regression analysis

indicated a strong positive relationship between the number of eggs laid and the emission of the constitutive volatiles ($F = 24.9$; $df = 1,5$; $P = 0.004$; $r^2 = 0.83$).

Lygus bugs that were caged in collection chambers in the absence of plants emitted the pheromone (*E*)-2-hexenyl butyrate (data not shown). Aldrich et al. (1988) previously reported that this compound accounts for about 70–80% of the total volatiles emitted from male and female *L. hesperus*. The compound was not detected in the volatile blend emitted by plants after the insects were removed, indicating that plants emitted little or no (*E*)-2-hexenyl butyrate.

Feeding by *L. hesperus* on maize seedlings induced the emission of (*Z*)-3-hexenyl acetate, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, indole, α-*trans*-bergamotene, (*E*)-β-farnesene, (*E*)-nerolidol, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Figure 1). Adults of both genders induced similar amounts of the same blend of volatiles from maize plants (data not shown; $t = 0.11$, $P > 0.05$).

Systemic Induction of Cotton Volatiles by Lygus Feeding. The systemic induction of plant volatile emission was evident when comparing insect-free control plants with plants that had *Lygus* adults enclosed on lower leaves that were outside of the VOC collection chamber ($t = 5.13$, $P < 0.05$; Figure 2). Systemically induced compounds included (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, (*E*)-4,8,dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*E*,*E*)-α-farnesene, and

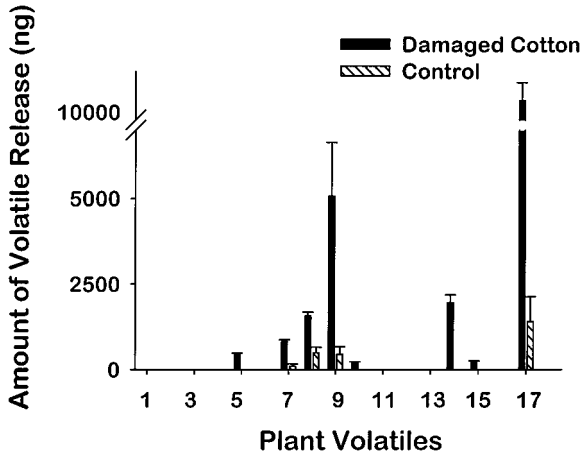


FIG. 2. Systemic induction of volatile emission from cotton plants by *Lygus hesperus* feeding. Volatiles were collected from portions of the plant not infested with insects or from a similar portion of control plants maintained free from insects. Volatile designations are same as described in Figure 1. Cumulative volatiles emitted during the photoperiod of four consecutive days were collected. Bars represent the mean + 1 SE; $N = 4$. Total volatile emissions: damaged cotton = 22536.7 ± 5394.3; control = 2448.1 ± 1159.4.

TABLE 1. EFFECTS OF STEM GIRDLING ON EMISSION OF SYSTEMICALLY INDUCED VOLATILES IN COTTON PLANTS INFESTED BY *Spodoptera exigua* LARVAE OR *Lygus hesperus* ADULTS

Treatment ($N \geq 5$)	Total volatiles (ng ± SE)[a]	dpm/g FW($\times 10^{-2} \pm$ SE)[b]		
		Labeled leaf	Small leaves	Apex
No girdle				
No insects, no $^{14}CO_2$	2448 ± 1159 a[c,d]	11 ± 6 b	4 ± 2 a	2 ± 0 a
S. exigua	15545 ± 4007 bc	10856 ± 1554 a	79 ± 28 b	188 ± 74 b
L. hesperus	22536 ± 5394 c[d]	ND[e]	ND	ND
Girdled				
No insects	2665 ± 1502 a	8760 ± 290 a	7 ± 0.5 a	8 ± 3 a
S. exigua	5045 ± 1089 a	11057 ± 1291 a	7 ± 1 a	14 ± 3 a
L. hesperus	6270 ± 1982 ab	8554 ± 1877 a	8 ± 2 a	20 ± 8 a

[a] Cumulative volatiles emitted during the photoperiod of 4 consecutive days.
[b] Disintegrations per min per gram fresh weight.
[c] Numbers in a column followed by the same letter indicate no significant differences (pairwise Tukey test, $P > 0.05$; volatiles: $F = 6.1$; $df = 5,40$; $P < 0.001$; labeled leaf: $F = 9.02$; $df = 4,23$; $P < 0.001$; small leaves: $F = 5.03$; $df = 4,23$; $P = 0.005$; apex: $F = 4.36$; $df = 4,23$; $P = 0.009$).
[d] Data obtained from Figure 2.
[e] Data not determined.

(*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. The same compounds were emitted systemically from cotton plants infested with *S. exigua* (Paré and Tumlinson, 1998; Rodriguez-Saona et al., 2001).

Girdling of cotton stems substantially disrupted phloem transport as observed by the movement of photosynthate in the plant. The transport of photosynthetically fixed $^{14}CO_2$ from a leaf below the girdled region to the apical portions of the plant was reduced by up to 90% compared to the nongirdled control plants (Table 1). This difference was observed for both apical tissue and small leaves near the apex of the plant. The girdling treatment was severe, such that live tissue was not readily detectable and stems had to be tied to stakes to remain upright. However, approximately 75% of the plants treated in this manner maintained turgid leaves above the stem girdle for at least one week, indicating that water could still move through the girdle to the top of the plant.

The systemic induction of volatile emission from cotton plants was markedly reduced for plants that had been heat-girdled around the stem (Table 1). The induced response of girdled plants was reduced by 60–70% compared with VOC emissions from nongirdled plants.

Induction of Maize Volatiles by Lygus *Salivary Gland Extracts*. Female glands were nearly twice the volume of male glands ($t = 3.59$; $P < 0.001$), with the mean volume for female salivary glands averaging 0.35 ± 0.04 mm^3 (mean ± SE, $N = 30$), while male salivary glands averaged 0.18 ± 0.03 mm^3 (mean ± SE, $N = 20$). The larger female glands were used for the bioassays.

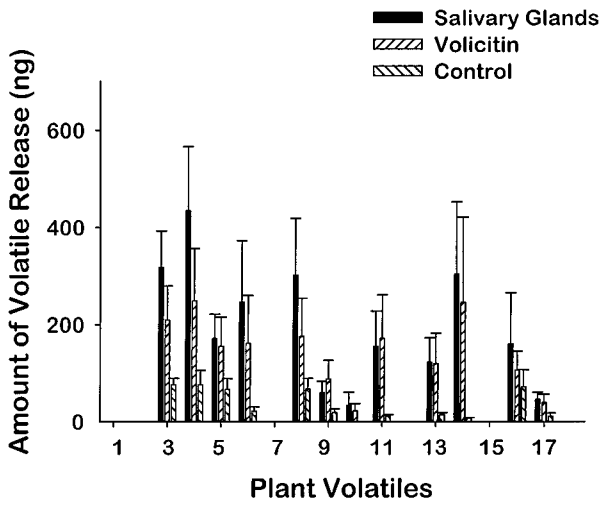


FIG. 3. Volatiles collected from maize seedlings incubated with distilled water (control), the caterpillar elicitor volicitin, or *Lygus hesperus* salivary gland extracts. Volatile designations are the same as described in Figure 1. Volatiles were collected for 6 hr starting at 10:00 hr. Bars represent the mean + 1 SE; $N = 5$. Total volatile emissions: salivary glands = 3124 ± 825.6; volicitin = 1959.9 ± 625; control = 576.8 ± 113.7.

Lygus salivary gland extracts induced the emission of the same blend of volatiles from maize seedlings as observed for live insects (Figures 1 and 3). Additionally, the volatile blend emitted was similar to the blend induced by the caterpillar elicitor volicitin (Figure 3).

DISCUSSION

Lygus hesperus feeding on cotton and maize plants induced the emission of plant volatiles (Figure 1) and, with the exception of the constitutive compounds stored in the cotton tissue glands, a similar induction of volatiles was caused by nymphs, mature males, and ovipositing females. Mature ovipositing females caused the emission of significant amounts of the constitutive volatiles from cotton, and the level of emission was closely associated with the number of eggs laid on a plant. In some cases, oviposition damage has been observed to activate induced responses in plants (Meiners and Hilker, 1997, 2000). Although our experiments could not distinguish whether oviposition alone induced the emission of the nonconstitutive volatiles, it can be concluded that mechanical damage to glands by oviposition contributed significantly to the emission of the constitutive volatiles. Female *L. hesperus* inserted their eggs inside the leaves, petioles,

and stems, causing rupture of glands adjacent to the oviposition site (Rodriguez-Saona, personal observation). Our results indicated that insect feeding per se was the predominant factor leading to the emission of the non-constitutive volatiles from cotton plants.

Lygus feeding induced the emission of a similar blend of volatiles as observed for caterpillars (Loughrin et al., 1995; Paré and Tumlinson, 1997a,b): In their experiments using ^{13}C-labeled CO_2, Paré and Tumlinson (1997a,b) demonstrated that these volatiles were synthesized de novo at the site of feeding. Kessler and Baldwin (2001) also reported a similar blend of volatiles was emitted from field-grown *Nicotiana attenuata* Torr. ex Wats plants infested by caterpillars or the piercing–sucking insect *Dicyphus minimus* (Heteroptera: Miridae). Similar to experiments with caterpillars feeding on plants (Röse et al., 1996; Paré and Tumlinson, 1998), *L. hesperus* feeding systemically induced the emission of a blend of plant volatiles that, with the exception of the constitutive glandular compounds, was qualitatively similar to the blend emitted from leaves that incurred direct feeding activity (Figure 2).

Heat-girdling of cotton stems significantly decreased the emission of systemically induced volatiles (Table 1). However, although not significant statistically, the insect-infested girdled plants consistently emitted greater quantities of volatiles than girdled plants kept free from insects (Table 1). Thus, translocation of the signal, presumably chemical, initiated by *Lygus* was attenuated but not completely blocked by disruption of stem phloem tissue. The girdling treatment also disrupted phloem transport, as indicated by the approximately 10-fold decrease in translocation of photosynthetically fixed $^{14}CO_2$ compared to nongirdled controls. However, as for volatile induction, a small but consistent amount of ^{14}C was detected in the apex of girdled plants. This continued low level of movement of phloem products across the wound site may be due to a transfer of phloem materials to the xylem at the site of girdling, followed by xylem transport to the upper parts of the plant. The fact that the upper leaves maintain their turgor subsequent to stem girdling confirms the flow of water in the xylem tissue. The difference in magnitude of the girdling effect on inhibiting ^{14}C translocation and blocking the systemic emission of volatiles may simply be due to a high potency of the systemic signal in inducing volatile emissions. In the case of systemin, a mobile signal molecule present in tomato, femtomole quantities of this polypeptide are sufficient to activate the systemic induction of proteinase inhibitors (Ryan and Pearce, 1998).

Although the nature of the elicitor has yet to be determined, the fact that *Lygus* salivary gland extracts induced the emission of volatiles from maize seedlings suggests the presence of an elicitor that triggers volatile emissions. Indeed, the blend of volatiles induced by *Lygus* salivary gland extracts was similar to the blend induced by treatment with authentic volicitin, which has been isolated from the oral secretions of caterpillars (Figure 3). Volicitin treatment apparently stimulates the octadecanoid pathway (Alborn et al., 2000), leading to the production of

jasmonic acid, a growth regulator known to stimulate VOC emissions from leaves (see Boland et al., 1998, for review). In fact, the same volatiles induced by *L. hesperus* (Figure 1) and *S. exigua* (Paré and Tumlinson, 1997a,b) in cotton are activated locally and systemically by exogenous treatment of methyl jasmonate, a volatile derivative of jasmonic acid (Rodriguez-Saona et al., 2001). Thus, while definitive determination of the elicitor in *Lygus* salivary glands is lacking, there is the possibility that the mechanism of volatile induction caused by *Lygus* feeding is similar to the response induced by volicitin.

In summary, we have shown that feeding by a phytophagous piercing–sucking insect induces the emission of plant volatile compounds in a manner comparable to the well-documented response observed for caterpillars. Similarities in the volatile blends emitted by both types of herbivores, by *Lygus* salivary gland extracts, and by the caterpillar elicitor volicitin, indicate that *Lygus* feeding activates the same biosynthetic pathway as caterpillars. Volicitin and other related compounds have been isolated from the regurgitate of various caterpillar species (Pohnert et al., 1999; Halischke et al., 2001). However, the amounts of these compounds for each species may vary (Mori et al., 2001), resulting in specificity in the plant's response to damage (De Moraes et al., 1998). Therefore, although caterpillars and piercing–sucking herbivores may share similar elicitor(s) in their salivary glands that induce a common plant-volatile signaling pathway, the plant response may vary with the amounts of elicitor(s) produced by these two different herbivores.

Herbivore-induced plant volatiles serve as important cues in host location by natural enemies (e.g., De Moraes et al., 1998; Kessler and Baldwin, 2001), and several parasitoids and predators attack the nymphs and adults of *Lygus* bugs (Ruberson and Williams, 2000). Whether or not plant volatile emission induced by *L. hesperus* feeding provides a foraging cue to these natural enemies remains to be determined.

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SEQUESTRATION OF GLUCOSINOLATES BY HARLEQUIN BUG *Murgantia histrionica*

ALIREZA ALIABADI,[1,*] J. ALAN A. RENWICK,[2] and DOUGLAS W. WHITMAN[3]

[1]*4160 Chemistry, Illinois State University*
Normal, Illinois 61790, USA

[2]*Boyce Thompson Institute*
Ithaca, New York 14853, USA

[3]*Behavior, Ecology, Evolution, & Systematics Section*
4120 Biological Sciences, Illinois State University
Normal, Illinois 61790, USA

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Abstract—*Murgantia histrionica*, the harlequin bug, is an aposematic pentatomid that feeds on toxic crucifer plants. By performing predator trials, we found that the bugs are distasteful to several species of bird predators. Given this, we tested the hypothesis that the bugs sequester toxins from the crucifer plants they feed on for use in defense against predation. We used high-pressure liquid chromatography for analyses and tested if *M. histrionica* sequesters toxic chemicals from its crucifer diet. We found that *M. histrionica* sequesters mustard oil glycosides, precursors to zootoxic mephitic nitriles, and that sequestration is characteristic of the plant species fed upon. Glucosinolate titers in *M. histrionica* bodies were 20–30 times higher than in their guts. We found that cabbage-fed *M. histrionica* had higher titers of cabbage glucosinolates than bugs that were fed on a cabbage diet and then switched to a diet of garden nasturtium. This indicates that *M. histrionica* immediately sequesters chemicals from whichever plant it feeds upon. The study shows that *M. histrionica* can sequester glucosinolates from its host plants for use in defense against predation and that the bugs can retain the glucosinolates for an extended period of time.

Key Words—Sequestration, glucosinolate, mustard oil glycoside, Hemiptera, Heteroptera, Pentatomidae, *Murgantia histrionica*, aposematism, Brassicaceae, crucifer.

*To whom correspondence should be addressed. E-mail: alia@citrus.ucr.edu

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INTRODUCTION

Insects are known to sequester plant secondary compounds, making them unpalatable to potential predators (Duffey, 1980; Blum, 1981; Bowers, 1990). By 1973, Rothschild listed 43 insect species thought to store host plant chemicals, and since then the number has grown substantially (Duffey, 1980; Bowers, 1990), with new examples added annually (e.g., Bowers and Stamp, 1997; Geuder et al., 1997; Dobler et al., 1998, 2000; Wink et al., 2000; Sime et al., 2000; Müller et al., 2001). However, to our knowledge, only two groups of true bugs (Heteroptera) have been shown via chemical analysis to sequester plant secondary compounds. Some Rhopalidae (*Jadera* and *Leptocoris*) sequester cyanoglucosides from seeds of Sapindaceae (Braekman et al., 1982; Aldrich, 1988), and some Lygaeidae (*Oncopeltus*, *Caenocoris*, *Spilostethus*, and *Lygaeus*) sequester cardenolides from Asclepiadaceae or Apocyanaceae (Von Euw et al., 1971; Duffey and Scudder, 1972; Scudder and Duffey, 1972), while another (*Neacoryphus*) sequesters pyrrolizidine alkaloids from *Senecio* (Asteraceae) (McLain and Shure, 1985).

The paucity of examples of sequestration among the Heteroptera is unexpected considering their abundance and feeding ecology. Indeed, well over 20,000 heteropteran species feed on plants (Schuh and Slater, 1995), and many of these specialize on chemically defended plant taxa (Aldrich, 1988; McGavin, 1993). Sequestration is most often seen in insect herbivores that specialize on toxic plants (Bowers, 1990). Furthermore, true bugs are often conspicuously colored (Schuh and Slater, 1995), a trait regularly associated with chemical defense (Pasteels et al., 1986; Guillford, 1990). Most Heteroptera expel odorous defensive secretions when disturbed, and these defensive secretions may account for the evolution of aposematic coloration in bugs. However, chemically defended insects often possess a two-component allomonal system consisting of an odorous, volatile secretion that startles, alerts, or warns potential predators, backed up by less volatile internal toxins that are often derived from host plants and that sicken predators after ingestion (Brower, 1984; Whitman, 1988). Furthermore, as Aldrich (1988) reported, some Heteroptera possess a bitter or offensive taste independent of any secretion. In other bug species, palatability to predators depends on diet (McLain, 1984). Taken together, this evidence suggests that sequestration of host plant compounds in the Heteroptera is probably more common than currently recognized and that many examples of sequestration await discovery (Aldrich, 1988).

An ideal candidate for sequestration of host plant compounds is the harlequin bug, *Murgantia histrionica* (Hahn) (fam. Pentatomidae). This agricultural pest (Metcalf et al., 1962; McPherson, 1982; McPherson and McPherson, 2000), native to Central America (Walsh, 1866; McPherson, 1982) exhibits many of the characteristics associated with the chemical defense syndrome (Pasteels et al., 1983; Whitman et al., 1985). They are large, diurnal, conspicuously colored with black and orange or red markings, slow moving, resistant to flying, and often

clumped, making them easy to find and capture, yet they have few predators (Ludwig and Kok, 1998). Harlequin bugs specialize on plants in the cabbage family (Brassicaceae), such as cabbage, broccoli, kale, turnip, radish, horseradish, mustard, rape, etc. (McPherson, 1982). Plants in this family often have a hot or sharp taste due to high concentrations of mustard oil glycosides, compounds known for their antifeedant and toxic properties (Louda and Mole, 1991; Zrybko and Rosen, 1997; Aliabadi and Whitman, 2001). Aldrich et al. (1996) recently detected aglycones of mustard oil glucosides in harlequin bugs, suggesting that *M. histrionica* obtained these compounds from its crucifer host plants.

As in virtually all pentatomids, *M. histrionica* possesses metathoracic scent glands (MTGs); however, the glands are much smaller than in most other members of this family (Aldrich et al., 1996), and they expel less secretion upon disturbance. Indeed, we were unable to obtain any liquid from the MTGs of many of the individuals we collected from the field or the laboratory (Whitman, unpublished). The MTG secretion contains primarily (2*E*, 6*E*)-octadienedial, with small amounts of (*E*)-2-octenal and (2*E*, 6*E*)-octadiene-1,8-diol diacetate (Aldrich et al., 1996). In addition, when squeezed, harlequin bugs expel a frothy fluid from the margins of the prothorax containing two odorous alkylmethoxypyrazines (Aldrich et al., 1996), a class of compounds thought to function as warning odors in a large number of chemically defended insects (Guilford et al., 1987; Kaye et al., 1989; Moore et al., 1990; Aldrich et al., 1997). The prothoracic fluid also appears to contain three thiocyanides (*sec*-butyl isothiocyanate, methylthiopropyl nitrile, and methylthiobutyl nitrile) (Aldrich et al., 1996), which are related to glucosinolates commonly found in cabbage (Fenwick et al., 1983). In this paper, we demonstrate that harlequin bugs sequester mustard oil glucosides from their crucifer host plants and that the specific compounds sequestered depend on the plant species fed upon. We also show that some birds reject harlequin bugs as prey.

METHODS AND MATERIALS

Insects. Harlequin bugs, *Murgantia histrionica* (Hahn), were collected from wild mustard plants in Clearlake, California, USA, in 1997 and thereafter cultured at room temperature in the laboratory at Illinois State University on cabbage, mustard, and kale.

Experiment 1—Single-Plant Diets. We established colonies of harlequin bugs on six different plant species: *Sinapis alba* (white mustard), *Crambe abysinnica* (crambe), *Armoracia rusticana* (horseradish), *Brassica oleracea* (cabbage), *Tropaeolum majus* (garden nasturtium), and *Buddleia davidii* (butterfly bush). For the first three, the plants and insects were raised in isolated outdoor plots at the ISU farm. For the remaining three, colonies of bugs were established in the laboratory and fed leaves of store-bought cabbage or garden-grown nasturtium or butterfly bush. Each colony remained on its single plant species diet for 4–9 weeks

(4 weeks on butterfly bush, after initial growth on cabbage), which, for all diets except butterfly bush, was long enough for the bugs to complete a generation. Bugs on butterfly bush lived, but did not grow or reproduce. The first four plant species are Brassicaceae, whereas nasturtium and butterfly bush are Tropaeolaceae and Loganlaceae, respectively. The three plant families are not closely related, but nasturtium contains a mustard oil glucoside, glucotropaeolin, as a major constituent. After 4–9 weeks, bugs from each diet were chemically analyzed for presence of glucosinolates.

Experiment 2—Effects of Host-Plant Transfer. In order to investigate the effects of host-plant transfer on potential sequestration, we analyzed the glucosinolate contents of bugs reared on two diets: cabbage for 6 months and then switched to nasturtium for either 3 or 6 days.

Experiment 3—Glucosinolate Titers in Guts and Bodies. To determine if glucosinolates were present in body tissues other than the gut, we raised harlequin bugs for 6 months on cabbage, then dissected out the digestive system, being careful not to contaminate the insect hemocoel with gut contents. We analyzed 20 guts and 20 degutted bugs for presence of glucosinolates.

Chemical Analysis. We extracted plants by immersing foliage (0.6–0.9 g fresh weight) in 5 ml boiling water for 10 min. Bugs were extracted in batches of 5 (ca. 0.3 g total weight) from each plant by quick freezing before immersion in boiling water for 10 min. We filtered the resulting aqueous extracts, then added these to individual DEAE Sephadex columns for enzymatic desulfation of the glucosinolates, according to the method of Minchinton et al. (1982). High-pressure liquid chromatography (HPLC) of the desulfoglucosinolates was conducted on a Hewlett Packard model 1100 apparatus equipped with a diode array detector. The column was a C-18 Luna 3μ, 150×4.6 mm (Phenomenex, Torrance, California, USA), with a flow rate of 0.75 ml/min. A solvent program of water and acetonitrile was used, with 100% H_2O from 0 to 5 min, a linear increase in acetonitrile to 35% at 35 min, and a linear increase to 100% acetonitrile at 40 min. Injections of 100 μl from 4 ml total sample were used, and the UV absorption of the eluate was monitored at 229 nm.

Frozen bug guts and degutted bodies were extracted in the same way in batches of 5, then analyzed by HPLC; the column was a semipreparative C-18 Luna 5μ, 250×10 mm (Phenomenex), with a flow rate of 1.8 ml/min. The same water/acetonitrile solvent gradient was used as for the analytical column.

We did not attempt quantitative analysis due to a lack of standard compounds; however, we made comparative estimates of quantities present in the bugs based on peak area units as a result of integration of the chromatographic peaks. We tentatively identified individual glucosinolates based on retention times, comparison with extracts of plants of known glucosinolate composition, and UV spectra.

Predator Trials. To determine if harlequin bugs were palatable to bird predators, we trained wild European starlings, *Sturnus vulgaris*, and house sparrows,

Passer domesticus, to come to a ground-level feeder (a 30- × 30-cm board) containing adult, larval, and pupal mealworms (*Tenebrio molitor*), and small pieces of raw pork. After 4 days of training, we began testing the birds by observing their behaviors from a blind, 3 m distant. We used harlequin bugs that had been reared on brown mustard, *Brassica juncea*. All insects in all tests were frozen and then thawed before use, and all tests were conducted on May 30, 1999, a cloudless, humid summer day. Except for trial 4, all trials ended about 4 min after the last control insect was removed from the feeder. Crickets (*Acheta domesticus*) were not offered during the training period, but were offered during the trials. Hence, our experimental design allowed us to test the responses of birds to known palatable prey (mealworms), novel palatable prey (crickets), and novel unpalatable prey (harlequin bugs). Trial 1 took place at 14:02 hr. Two boards were placed 10 cm apart. Board 1 contained 8 adult crickets; board 2 contained 8 adult harlequin bugs. Trial 2 took place at 14:38 hr. A single board was placed out containing 7 adult, 7 larval, and 1 pupal mealworm, 7 pieces of pork, and 7 adult harlequin bugs. Trial 3 took place at 15:20 hr. We placed 14 adult and 13 larval mealworms, 6 pieces of pork, and 19 adult harlequin bugs onto a single board. Trial 4 took place at 15:35 hr. We placed 16 adult harlequin bugs onto a single board; this trial continued for 1 hr. Trial 5 took place at 17:50 hr. We placed 9 adult and 11 larval mealworms, 8 adult crickets, and 12 adult harlequin bugs onto one board.

RESULTS

Chemical Analysis of Single-Plant Diets. Insects from each of the four colonies of harlequin bugs that fed on a single crucifer diet and the bugs that fed on nasturtium showed qualitative differences in glucosinolate content, matching, for the most part, the chemistry of the plant species fed upon (Table 1). Insects fed cabbage contained sinigrin and glucobrassicin (Figure 1), the two most prominent glucosinolates in cabbage. White mustard contained glucosinalbin, which eluted at 15.8 min, and this matched the major peak (at 15.8 min) from extracts of the harlequin bugs fed on this plant (Figure 2). Extracts of crambe plants revealed sinigrin at 13.2 min and additional glucosinolates, probably gluconapin at 13.7 min, progoitrin at 17.7 min, and hydroxyglucobrassicin at 18.3 min. An additional compound at 25.6 min matched the retention time and UV spectrum of gluconasturtiin. The adult harlequin bugs fed on crambe showed three peaks at 13.1, 13.6, and 17.6 min, matching the retention times and UV spectra of sinigrin, gluconapin, and progoitrin (Figure 3). Insects fed horseradish contained sinigrin, which eluted at 13.5 min and an unknown at 28.0 min. Analysis of adult harlequin bugs raised on horseradish indicated the presence of sinigrin at 13.6 min, but not the unidentified peak at 28.0 min (Figure 4). Glucotropaeolin was the major glucosinolate in garden nasturtium (28.5 min), and in bugs fed this noncrucifer, glucotropaeolin eluted at the same time (Figure 5). Butterfly bush contained no glucosinolates,

TABLE 1. COMPOUNDS DETECTED IN SIX DIFFERENT PLANT SPECIES AND IN HARLEQUIN BUGS RAISED ON THEM

Plant species	Compounds detected in	
	Food plant	Harlequin bug
Cabbage (*Brassica oleracea*)	sinigrin (21.5)[a], glucobrassicin (≈31.0)	sinigrin (21.5), glucobrassicin (≈30)
White mustard (*Sinapis alba*)	glucosinalbin (15.8)	glucosinalbin (15.8)
Crambe (*Crambe abyssinica*)	sinigrin (13.2), gluconapin (13.7), progoitrin (17.7), hydroxyglucobrassicin (18.3), gluconasturtiin (25.6)	sinigrin (13.1), gluconapin (13.1), progoitrin (17.5)
Horseradish (*Armoracia rusticana*)	sinigrin (13.5), unknown (28.0)	sinigrin (13.6)
Garden nasturtium (*Tropaeolum majus*)	glucotropaeolin (28.5)	glucotropaeolin (28.5)
Butterfly bush (*Buddleia davidii*)	none detected	sinigrin (21.5)

[a] HPLC Retention time (minutes).

but sinigrin and other glucosinolates were present in the harlequin bugs fed this control for 4 weeks.

Host-Plant Transfer. Adult harlequin bugs fed cabbage for 6 months, then transfered to nasturtium for 3 days or 6 days, contained sinigrin, glucobrassicin,

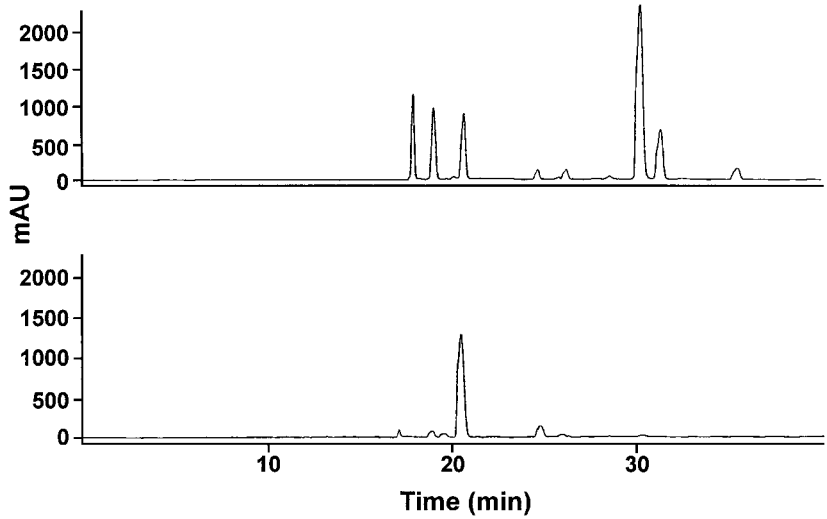


FIG. 1. Harlequin bugs (bottom) fed cabbage (top). Peak at 21.5 min (top) is sinigrin and peak at 31.0 (top) is glucobrassicin.

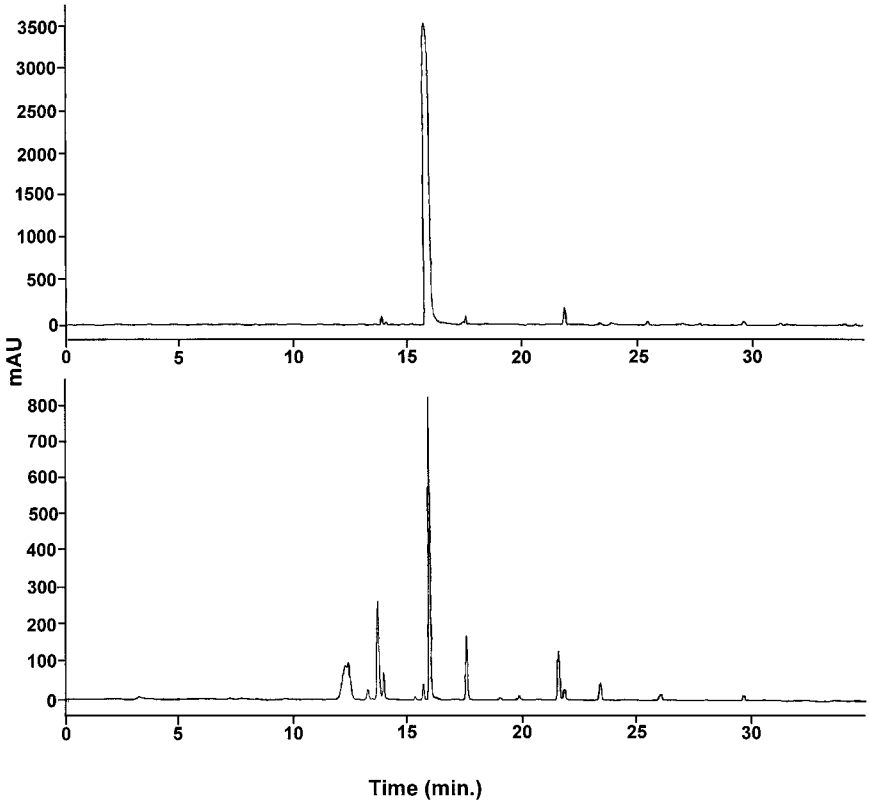


FIG. 2. Harlequin bugs (bottom) fed white mustard (top). Glucosinalbin peak appears at 15.87 min (top).

and glucotropaeolin, suggesting that the bugs had acquired substances from both diets. In animals fed nasturtium for 3 days (Figure 6a), sinigrin was the major peak whereas in animals fed nasturtium for 6 days (Figure 6b), sinigrin was reduced and glucotropaeolin was the major peak.

Glucosinolate Presence in Degutted Bugs. Harlequin bug guts and degutted bodies contained both sinigrin and glucobrassicin, the two major glucosinolates in cabbage, but the bug bodies had higher concentrations of both compounds than the bug guts. In addition, both the guts and bodies contained sinigrin in higher concentrations than glucobrassicin, despite the predominance of glucobrassicin in cabbage (data not shown).

Predator Trials. At least six sparrows and four starlings visited the feeders during the course of the five trials. Normally, two to three birds fed at the feeder at any one time, with six individuals feeding simultaneously on one occasion. In all

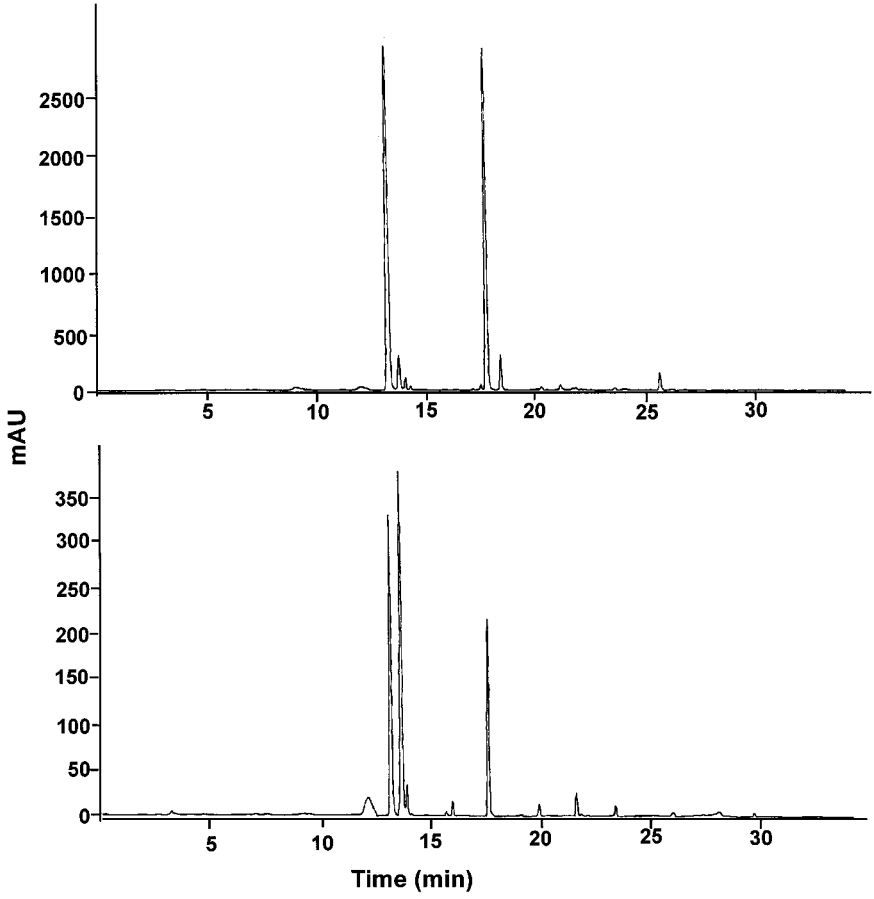


FIG. 3. Harlequin bugs (bottom) fed crambe (top). Sinigrin appears at 13.2, gluconapin at 13.7, progoitrin at 17.7, hydroxyglucobrassicin at 18.3, and gluconasturtiin at 25.6 min (top).

trials, birds arrived at the feeders within 6.5 min of the start of the trial. Individual birds normally remained at the feeder for less than 2 min, during which time they took four to seven prey items, then flew away. In trials 1, 2, 3, and 5, all control food items (crickets, pork, and mealworms) were removed from the feeder by 14, 7, 8, and 20 min, respectively.

During the trials, birds demonstrated a strong preference for crickets, pork, and larval, pupal, and adult mealworms, but rejected nearly all harlequin bugs (Table 2). Over the course of the five trials, the birds removed all 91 control (palatable) food items offered, but only 3 of the 62 harlequin bugs offered. On

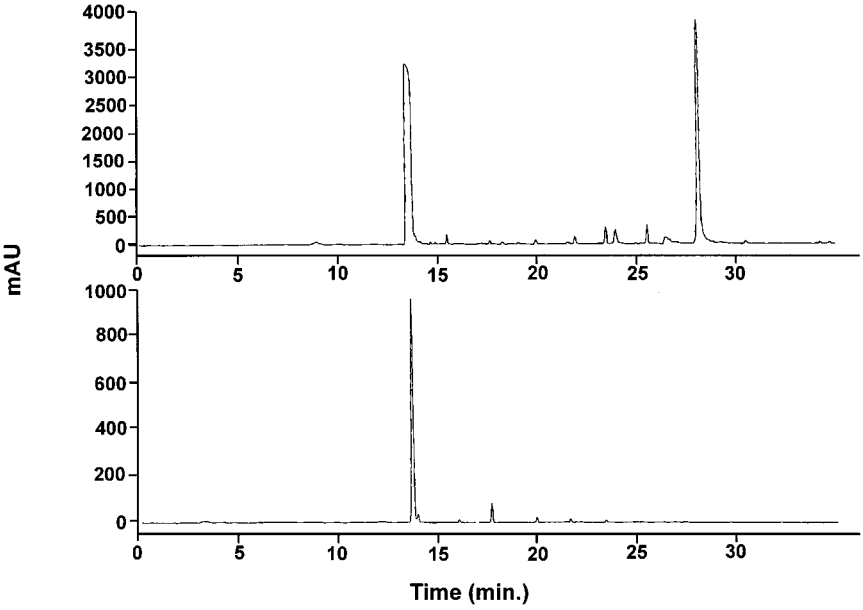


FIG. 4. Harlequin bugs (bottom) fed horseradish (top). The two major peaks for horseradish (sinigrin and unknown, respectively) appear at 13.5 and 28.0 min.

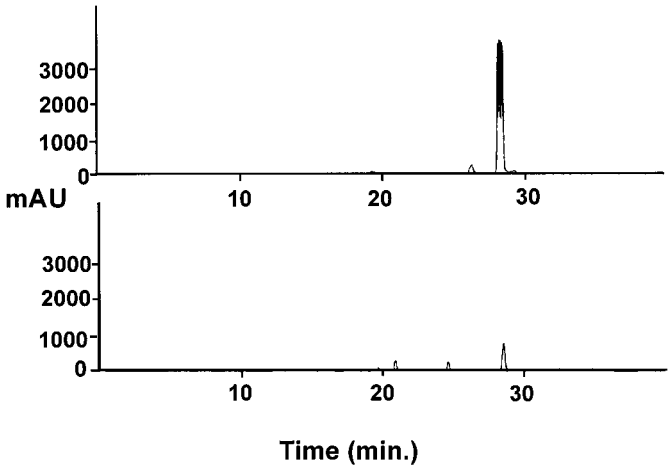


FIG. 5. Harlequin bugs (bottom) fed garden nasturtium (top). The major glucosinolate for this plant is glucotropaeolin (28.5 min).

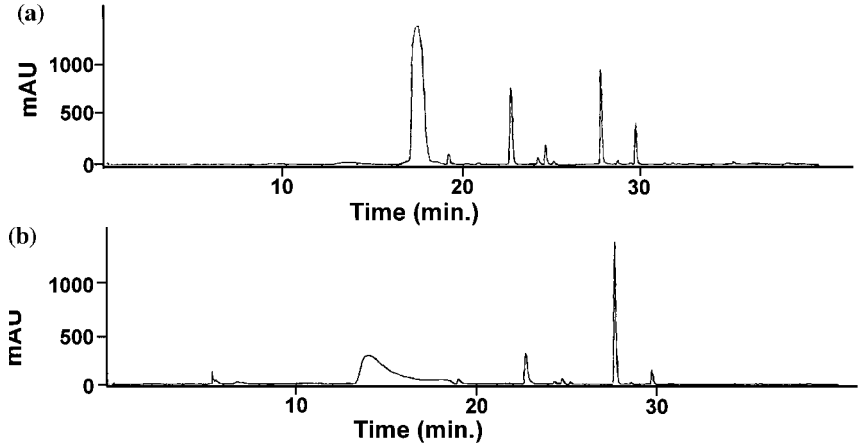


FIG. 6. (a) Harlequin bugs fed cabbage for 6 months, then transferred to garden nasturtium and allowed to feed for 3 days. Sinigrin (17.5 min), glucotropaeolin (27.5 min), and glucobrassicin (29.5 min). (b) Harlequin bugs fed cabbage for 6 months, then transferred to garden nasturtium and allowed to feed for 6 days. Sinigrin (14.0 min), glucotropaeolin (27.5 min), and glucobrassicin (29.5 min).

three occasions, we observed a sparrow pick up, then drop, a harlequin bug. On three other occasions, we found harlequin bugs on the ground away from the feeder, suggesting that they had been flung or knocked away by birds. In trials 1, 2, 3, and 5, many birds returned to the feeder after all control prey items had been taken. These birds moved about the feeder and inspected the remaining harlequin bugs (cocked or lowered their heads), but did not feed on them. When only harlequin bugs were offered (trial 4), starlings inspected the feeder 4 times and sparrows inspected 12 times, during a 1-hr period, but no harlequin bug was attacked.

TABLE 2. PREY REMOVED BY WILD STARLINGS AND HOUSE SPARROWS FROM A FEEDER

	Control items (N)		Harlequin bugs (N)	
Trial	Offered	Removed	Offered	Removed
1	8	8	8	1
2	22	22	7	0
3	33	33	19	1
4	0	0	16	0
5	28	28	12	1
Total prey	91	91	62	3

DISCUSSION

The results suggest that harlequin bugs sequester glucosinolates from crucifer host plants and thereby gain defensive benefits. To our knowledge, this is the second example of sequestration in the large family Pentatomidae, the fourth example of sequestration of plant toxins among the Heteroptera, and the third example of sequestration of crucifer glucosinolates by an insect (Aliabadi and Whitman, 2001; Müller et al., 2001). Larvae of the large white butterfly, *Pieris brassicae*, feed on crucifers and apparently sequester glucosinolates, making the adults the most toxic of all British Pieridae (Alpin et al., 1975). In contrast, *P. brassicae* caterpillars reared on artificial diets are readily consumed by birds, suggesting that they lose their repellent qualities when denied a source of glucosinolate.

The chemical analysis of whole bodies demonstrates that harlequin bugs are capable of acquiring a range of glucosinolates from their diet and that the specific compounds obtained reflect the plant species consumed. Bugs fed cabbage acquire cabbage glucosinolates. Bugs fed crambe contain glucosinolates specific to crambe, and bugs fed garden nasturtium contain the glucosinolate specific to that plant. These substances can be acquired from either plants in the cabbage family or from those in other families (e.g., Tropaeolaceae), if they contain glucosinolates.

The host plant transfer studies show that bugs that fed on garden nasturtium for only 3 days contained appreciable quantities of glucotropaeolin, a glucosinolate specific to this plant. These studies also suggest that the bugs can retain acquired glucosinolates for some time. For example, bugs transferred from cabbage to garden nasturtium, a plant that contains no sinigrin, still possessed a high titer of sinigrin after 6 days of feeding only on nasturtium. Because 6 days is long enough for the bugs to empty their guts of cabbage sap, the presence of sinigrin implies sequestration. Indeed, indirect evidence suggests long-term storage of glucosinolates: the adult bugs fed 4 weeks on butterfly bush, a plant devoid of glucosinolates, still contained small amounts of sinigrin, probably held from the time when they were originally reared on cabbage.

The presence of glucosinolates in whole body extracts of harlequin bugs does not necessarily demonstrate sequestration, because the glucosinolates may have been present only in the gut. True sequestration normally requires that the substance move out of the gut into other body tissues. In the case of cabbage-fed insects, sinigrin was found in both the gut and degutted body, but nondigestive tissues contained much higher concentrations of glucosinolates ($\sim$20–30 $\times$ greater) than guts, indicating that these compounds are concentrated in the body. The fact that sinigrin was not detected in insects fed white mustard or garden nasturtium (Table 1) suggests that the bugs do not synthesize this substance autogenously.

It is not known how the harlequin bug obtains the enzymes necessary to break down glucosinolates. Cruciferous plant tissues contain myrosinase, an enzyme that

degrades glucosinolates into volatile toxins (Fenwick et al., 1983). Therefore, the harlequin bug could sequester myrosinase along with glucosinolates, manufacture the enzyme, or do both.

In our experiments, sparrows and starlings did not consume harlequin bugs. This feeding aversion could have arisen via several mechanisms, including neophobia (Coppinger, 1970; Schlenoff, 1984; Sargent, 1990), dietary conservatism, innate or learned fear of aposematic color patterns (Guilford, 1990), or distasteful external chemicals such as MTG or prothoracic gland secretions (Whitman et al., 1990; Aldrich, et al. 1996). However, it is possible that the birds were deterred, in part, by the presence of plant-derived glucosinolates, because these compounds are known to be distasteful, aversive, or toxic to a wide range of predators, including vertebrates (Aliabadi and Whitman, 2001). Furthermore, insects that contain sequestered glucosinolates are generally shunned by predators. Finally, our observations argue against neophobia, dietary conservatism, or innate fear of warning patterns, because: (1) the birds readily attacked crickets the first time offered (trial 1); and (2) during the feeding trials, we observed birds picking up harlequin bugs in their beaks, and three bugs disappeared, presumably eaten or at least carried away by birds. The birds were not deterred from sampling the bugs; hence rejection was probably based on taste.

Although this is only the fourth known example of sequestration in the true bugs, this phenomenon is probably common, given the large number of warningly colored bugs that feed on chemically defended plants.

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PYRROLIZIDINE ALKALOIDS FROM *Senecio jacobaea* AFFECT FUNGAL GROWTH

W. H. G. HOL[1,2,*] and J. A. VAN VEEN[1,2]

[1]*Institute of Evolutionary and Ecological Sciences*
Leiden University
Kaiserstraat 63 2300 RA
Leiden, The Netherlands

[2]*Netherlands Institute for Ecological Research*
Postbox 40 6666 ZG, Heteren, The Netherlands

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Abstract—We investigated the growth-reducing effects of pyrrolizidine alkaloids (PAs) from *Senecio jacobaea* on nine plant-associated fungi (five strains of *Fusarium oxysporum*, two of *F. sambucinum*, and two of *Trichoderma* sp). Fungal growth was monitored on water agar media containing different concentrations of monocrotaline, retrorsine, or a purified extract of PAs from *S. jacobaea*. The growth rate of six strains was inhibited by PAs at the highest test concentration (3.33 mM), with the magnitude of the inhibition (7–35%) being dependent upon the specific fungus-PA interaction. In general, the PA extract caused the largest inhibition. However, the fungi isolated from *S. jacobaea* were positively affected by the PA extract (7–9%). Retrorsine N oxide was as effective as retrorsine in its inhibition of mycelium growth.

Key Words—*Senecio jacobaea*, pyrrolizidine alkaloids, anti fungal activity, retrorsine, N oxide, monocrotaline.

INTRODUCTION

Members of the plant genus *Senecio* produce a wide range of secondary metabolites. The best known are the pyrrolizidine alkaloids (PAs), which are also found in other genera of the Asteraceae such as *Eupatorium*, as well as in the Boraginaceae, Fabaceae, and Orchidaceae (Hartmann, 1999). PAs are ester alkaloids that can be found in as plants tertiary amines and their N oxides. In *Senecio*, PAs are synthesized in the roots as N oxides (Hartmann and Toppel, 1987),

*To whom correspondence should be addressed. E-mail: hol@rulsfb.leidenuniv.nl

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and the corresponding tertiary PAs are regarded as degradation products of N oxides.

In *Senecio* species, 124 different PA structures have been described (Hartmann and Witte, 1995), and in a single species like *Senecio jacobaea* L., up to 14 PAs can be found (Witte et al., 1992). In addition to possessing a large variation of PA structures, *Senecio* species vary greatly in the amount of PAs produced, a trait that has been shown to have a genetic component (Vrieling et al., 1993).

The major function of PAs is assumed to be defense against herbivores. PAs cause severe liver damage in many mammalian species (Mattocks, 1986), sometimes resulting in death (Molyneux et al., 1991). PAs may deter generalist insect herbivores (Van Dam et al., 1995) or have a negative influence on their growth (Vrieling et al., 1991). Specialist herbivores are sometimes attracted by PAs and sequester them for their own defense (Boppré, 1986; Schneider, 1987; Hartmann and Witte, 1995). Adapted insects store plant-derived PAs preferably as N oxides. N oxidation is believed to be a detoxification mechanism (Cheeke, 1994).

The target of PAs in mammals is cytochrome P-450 (Huan et al., 1998). In vitro assays have demonstrated that PAs can interfere with neuroreceptors (Schmeller et al., 1997) and protein synthesis (Wink et al., 1998). Moreover, recombinagenic activity of integerrimine, a PA from *Senecio*, has been detected in somatic cells of *Drosophila melanogaster* (Campesato et al., 1997).

The potential role of insect herbivores has received a great amount of attention in studies attempting to understand the selective forces behind PA diversity. The arms race between plant and attacker is hypothesized to lead to a continuous circle of adaptation by both the attacker and the producer, resulting in the continuous development of new defense compounds produced by plants (Ehrlich and Raven, 1964). However, pathogens such as fungi and bacteria can also be a threat to plant fitness and, therefore, may also influence the selection of defense mechanisms.

Little is known about the potential of PAs to inhibit fungal growth, and has, to date, been reported only for PAs from *Heliotropium* (Jain and Sharma, 1987; Marquina et al., 1989; Reina et al., 1995, 1997). It is unknown to what extent defense against plant pathogenic fungi has attributed to the diversity of PAs in *Senecio*. In this study, a range of plant-associated fungi was tested for their sensitivity to pyrrolizidine alkaloids from *S. jacobaea* and *Crotalaria spp*. Experiments were performed to address the following questions: (1) Do PAs affect fungal growth? (2) Are all PAs equally effective? (3) Do fungi differ in their sensitivity? (4) Are fungi from *S. jacobaea* adapted to PAs from this plant species? (5) Are N oxides equally toxic as bases?

METHODS AND MATERIALS

Experimental Design. The effects of different concentrations of PAs were tested on nine fungal strains in a block design with 10 replicates. In a second

experiment, the effects of retrorsine and retrorsine N oxide were tested on three fungal isolates in a block design with ten replicates.

PAs. Three pure PAs and a mixture of PAs (PA bulk) were used. Monocrotaline (99% pure), retrorsine (99.8% pure), and retrorsine N oxide (>99% pure) were purchased from Sigma-Aldrich (Schnelldorf, Germany). The PA bulk was extracted from leaf material of *S. jacobaea* using a modified version of the method developed by Koekemoer and Warren (1951). Dried milled plant material (1 kg) was soaked in methanol for 24 hr. The filtered methanol solution was evaporated, and the residue was dissolved in sulfuric acid (0.25 M). Chlorophyll and waxes were removed by repeated extraction with petroleum ether and diethylether. The N oxide forms of alkaloids were reduced to tertiary alkaloids by adding zinc dust to the acidic solution. The solution was filtered and brought to pH >10 by adding ammonia. The reduced PAs were extracted by repeated washing with dichloromethane. The composition of the evaporated dichloromethane washing was determined on a GC (HP 6890, 30 m × 0.25 μm, HP-1). The conditions were as follows: injector 250°C, temperature programme 0-22-5-250, split mode 1–30, carrier gas N_2 at 0.9 ml/min, pressure 56 kPa; detector FID. The main PAs (>10% of the total amount of PAs) of the PA bulk were senecionine (12%), seneciphylline (22%), jacobine (24%), and jaconine (24%). The PA content of the extract was 80%, as determined by a color reaction. This was a modification of a method developed by Mattocks (1967), who used monocrotaline (99% pure) as an external standard. To control for the 20% impurity, the crude PA extract was redissolved in dichloromethane and extracted with sulfuric acid. There was residue left in the dichloromethane that did not contain PAs. To check whether the PA bulk extract contained fungal growth-reducing compounds, other than PAs, this residue was included as a treatment, hereafter referred to as the "non-PA fraction." All PAs were dissolved in phosphate-buffered 50% methanol solution (pH 6) before mixing with the fungal growth medium.

TABLE 1. NAMES, ORIGINS AND MAINTENANCE OF FUNGAL SPECIES

Code[a]	Species	Host plant	Origin	Storage
F. oxy Sjac1	*Fusarium oxysporum*	*Senecio jacobaea*	The Netherlands	−80°C
F. oxy unk	*Fusarium oxysporum*	Unknown	Unknown	PDA[b]
F. oxy Care	*Fusarium oxysporum*	*Carex arenaria*	The Netherlands	−80°C
F. oxy Svul	*Fusarium oxysporum*	*Senecio vulgaris*	Switzerland	PDA
F. oxy Sjac2	*Fusarium oxysporum*	*Senecio jacobaea*	The Netherlands	−80°C
F. sam Lsax1	*Fusarium sambucinum*	*Leontodon saxatilis*	The Netherlands	−80°C
F. sam Lsax2	*Fusarium sambucinum*	*Leontodon saxatilis*	The Netherlands	−80°C
T. sp Care	*Trichoderma* sp.	*Carex arenaria*	The Netherlands	PDA
T. sp unk	*Trichoderma* sp.	Unknown	Unknown	−80°C

[a] Codes are abbreviations of species names and hostplants. Different numbers refer to different isolates.
[b] PDA = potato dextrose agar.

Fungal Isolates. Nine plant-associated fungal isolates were used (Table 1). Two fungi (taken from the NIOO-CTO collection) originated from unknown host plants. The seven other isolates were collected from dune plants, three from roots of PA producing plants (*S. jacobaea* and *S. vulgaris*), and four from non-PA-producing plants (*Leontodon* sp. *Carex* sp.).

To isolate the fungi, 1-cm pieces of roots were surface sterilized with natriumhypochloride 1% for 10 sec. The root pieces were placed on malt pepton medium, and fungi were isolated from the mycelium that developed on the agar. Isolated fungi were grown on potato dextrose agar. As inoculum for the first experiment, six agar plates were used with 14-day-old cultures of each fungus. For each PA, the inoculum originated from two plates to minimize variation within the treatment. In the second experiment, a subset of three from the nine isolates were used.

Bioassay. Water agar (Oxoid) was sterilized for 20 min at 100 kPa and 120°C and cooled to 50°C, after which the filter-sterilized alkaloid solutions were added. Segmented Petri dishes (Omnilabo, Breda, The Netherlands) were filled with water agar containing either the PA dissolvent only or the dissolvent with 3.33, 0.33, or 0.033 mM alkaloids. These concentrations are within normal plant PA range for *Senecio*. The PAs were tested in separate Petri dishes. For the PA mixture extracted from *S. jacobaea*, a molecular weight of 352 was assumed. This was based on the average molecular weights of senecionine, seneciphylline, jacobine, and jaconine, which were dominantly present in the extract. The 0.033 mM treatment of PA bulk was replaced by the non-PA fraction of the bulk. Fungal plugs (1 cm diam.), taken from the margins of a growing colony, were placed in the central corner of each compartment at the start of the experiment. Petri dishes were stored in darkness at 20°C. Radial growth of the fungal mycelium was measured daily in millimeters. The experiment lasted 30 days, at which time most dishes were completely covered with mycelium. In the monocrotaline treatment, three species did not grow for unknown reasons; no mycelium growth was observed on the PA agar and the control agar. Therefore, data are shown for six fungi instead of nine. In the second experiment, three treatments were present in each Petri dish: retrorsine (3,33 mM), retrorsine N oxide (3,33 mM), and a control with dissolvent.

Statistics. Due to the large variation in fungal growth among dishes, fungal growth rate was chosen as the experimental parameter. Growth rate was estimated as the slope of the linear relation between mycelium radius (mycelium radius >1 mm) and days after inoculation. Comparisons of means of the effects of different PA concentrations were tested with Friedman and Wilcoxon tests for related samples. The growth rate of a treatment was compared to the growth rate of the control in the same Petri dish. For comparisons of the effect of different PAs, mycelium growth rates relative to their controls were compared with Kruskal-Wallis and Mann-Whitney tests for unrelated samples. For all tests SPSS 8.0 was used (SPSS Inc., 1998).

RESULTS

PAs had a variable effect on fungal growth (Figure 1). For several fungus–PA combinations, the growth rate on agar with PA was lower than on the control. In total, six fungi showed reduced growth on agar with PAs. However, not all PAs had the same effect. The magnitude of inhibition depended on the fungus–PA combination. *Trichoderma* sp. was most sensitive, with a relative growth rate of 65% on agar with retrorsine or PA bulk. *F. oxysporum* Sjac was more inhibited by monocrotaline than by the other two PAs.

Mycelium growth was not always negatively influenced by the PA treatments. Five fungi were stimulated at intermediate concentrations of the PA bulk, and two fungi were stimulated even at the highest concentration of PA bulk (Figure 1). Both of these latter isolates were isolated from *S. jacobaea*. The non-PA fraction of the bulk, which replaced the 0.033 mM in the PA bulk, did not show a difference from the control, except for *F. sambucinum* Leo, which was inhibited by this bulk control. However, the inhibition by the 3.33 mM PA bulk was significantly stronger.

Retrorsine N oxide was as effective as retrorsine (Figure 2) in the inhibition of mycelium growth.

DISCUSSION

To our knowledge, this is the first time that PAs from *S. jacobaea* have been shown to inhibit fungal growth. PAs from *Heliotropium* have shown antifungal effects in different assays. Jain and Sharma (1987) demonstrated the inhibition of two fungal species, *Curvularia lunata* and *Fusarium moniliforme* by lasiocarpine N oxide by using an in vitro paper disk diffusion assay. Marquina et al. (1989) also used an agar diffusion method to show growth inhibition of *Aspergillus niger* by lasiocarpine. Reina et al. (1995, 1997) cultured *Fusarium* sp. on potato dextrose agar to which lasiocarpine, 3′-acetyltrachelanthemine, floridinine, or europine were added. *Fusarium moniliforme* was most sensitive to this treatment, with 49% mycelium growth inhibition at a concentration of 1.5 mM 3′-acetyltrachelanthamine. PAs from *Heliotropium* species differ in structure from those of *Senecio*. Retrorsine and monocrotaline are macrocyclic diesters, while the *Heliotropium* alkaloids are open chain diesters.

In most cases, a dose effect is visible, although in a few exceptional cases the lower concentration showed a greater inhibition than observed for the highest concentration, e.g., *F. oxysporum* Svul on monocrotaline. In some cases, the intermediate concentration stimulated fungal growth, whereas the highest concentration was inhibitory, e.g., *F. oxysporum* Care on PA bulk. The concentrations at which most of the inhibitory effects were found (3.33 mM and 0.33 mM) are comparable to PA concentrations found in plant tissue (3–0.3 mM wet weight) under natural

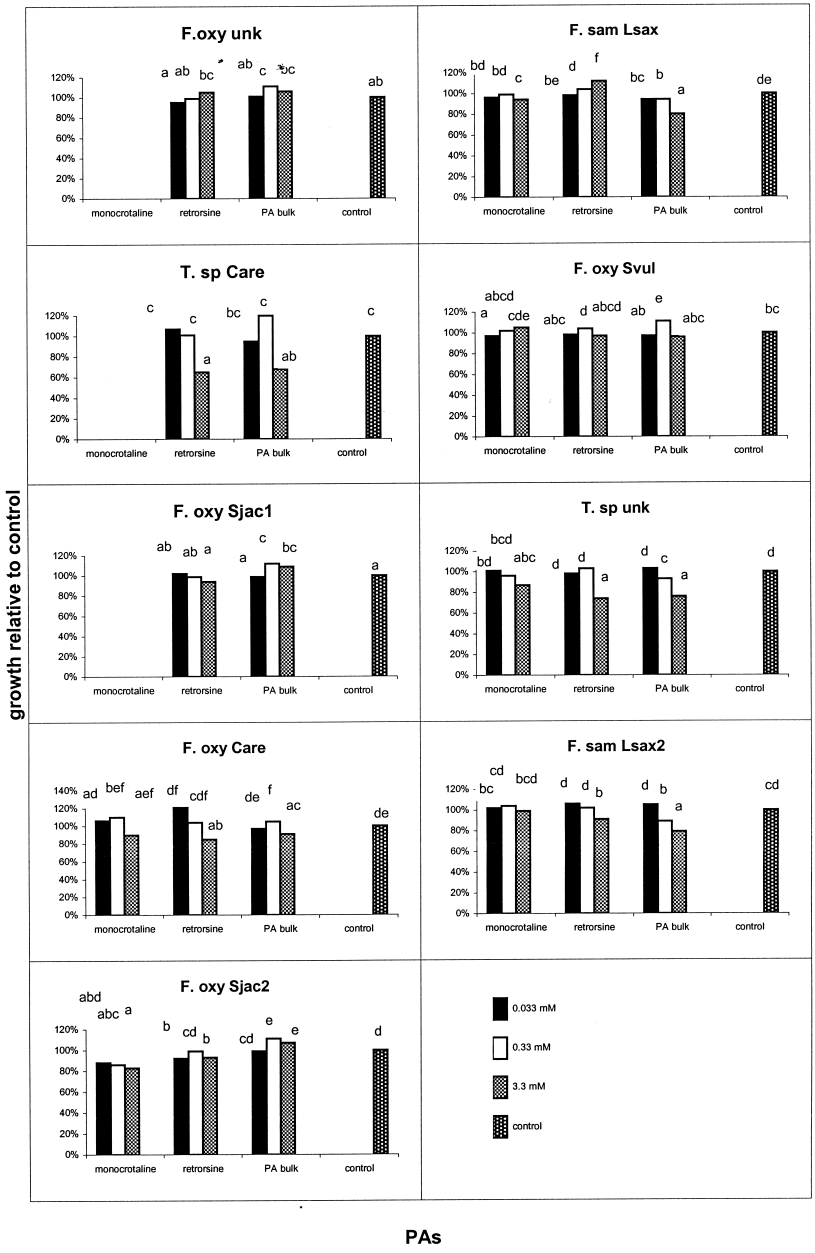


FIG. 1. The growth rate of nine fungi on medium with PAs at three different concentrations compared to the control. Different letters indicate a significant difference ($P < 0.05$, $N = 10$)

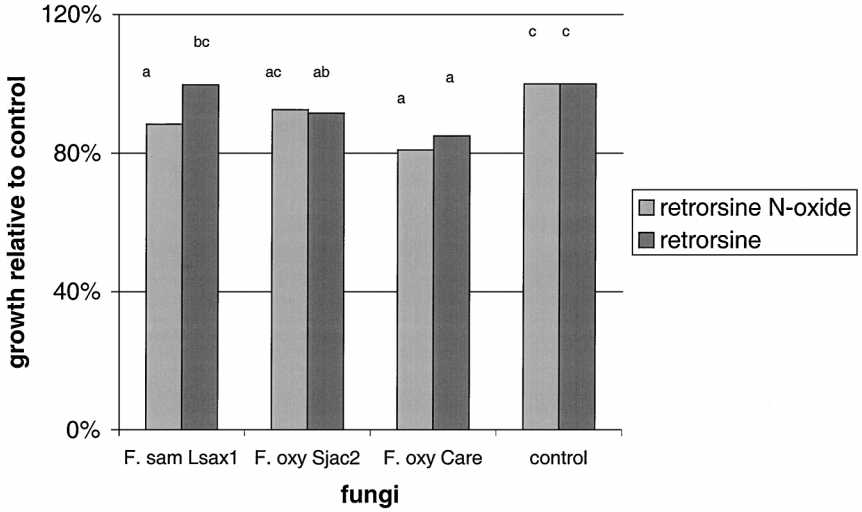


FIG. 2. The growth rate of three fungi on agar with retrorsine and retrorsine N oxide. Different letters indicate a significant difference ($P < 0.05$, $N = 10$)

conditions (Hol, unpublished results). We can only speculate about PA concentrations in the rhizosphere outside the root tissue. Low amounts of PAs have been detected in soil around the roots (E. van der Meijden, personal communication). Hartmann et al. (1989) did not detect PAs in the medium of in vitro cultured *Senecio vulgaris* roots. Similarly, Sander and Hartmann (1989) found no release of PA N oxide in the culture medium. Nevertheless, it is possible that, in soil, sloughing root cells with their PAs form a protective layer.

The tested PAs never totally arrested fungal growth. The growth rate was decreased for some fungi, while others exhibited an initial delay in mycelium growth followed by a normal growth rate. Such growth inhibition or delay may change the competitive abilities of pathogenic and nonpathogenic fungi in vivo, give the plant extra time to raise other defenses, or allow the plant root to escape the fungal attack. With regard to this last possibility, it is interesting to note that a vulnerable root tip can move 4 mm out of reach within 24 hr (Asplund and Curtis, 2001). Due to the artificial nature of our system, this study cannot be interpreted as definite evidence for the protection of plants by PAs. The types of hyphae growing in soil or on fungal growth medium may be very different. Aerial mycelium may be dominant in the Petri dish but not in soil.

pH was a factor in our experiment, but may be less important in plants, which are well buffered. Originally, the experiment was carried out without buffer in the medium. PAs increased the pH of the medium, potentially affecting fungal growth. However, repetition of the experiment, using a buffered system, gave

the same result, indicating that pH could not explain the observed fungal growth effects.

Not all tested compounds showed the same activity. Monocrotaline had a smaller spectrum of activity than retrorsine and the PA bulk. PA bulk was the most active. Growth-reducing effects from compounds other than PAs in *Senecio* spp. have been shown before, such as essential oil of *Senecio graveolens* (Perez et al., 1999), and β-sitosterol from *Senecio lyratus* (Kiprono et al., 2000). As the non-PA fraction of the bulk in general did not show an effect on fungal growth, it seems unlikely that fungal growth reducing compounds other than PAs were present in the extract. Therefore, despite the fact that the extract was not pure, the growth reducing effects are probably attributable to PAs only.

Several explanations are possible for the larger activity of the PA bulk. Combinations of compounds may be more effective than a single compound. We were not able to test for synergistic effects of different PAs in the extract, because not all the alkaloids from the extract were available in pure form. Besides possible synergistic effects, we cannot exclude the possibility that the stronger effect of PA bulk is due to other PAs, like senecionine and jacobine, which may be more active than retrorsine and monocrotaline. Furthermore, it should be pointed out that the PA composition of aboveground plant parts, which we used as the basis for the PA extract, is more diverse than that found in the roots. Tinney et al. (1998) found up to 93% senecionine and its stereoisomer integerrimine in the roots of reproducing *S. vulgaris*. In other plant parts, this varied between 40 and 55%. Therefore, the composition of the PA mixture was probably more diverse than the PA mixture that root-associated fungi in *S. jacobaea* will meet under natural conditions.

Remarkable differences in fungal sensitivity were observed in relation to the host plant. The relative resistance of fungi isolated from *S. jacobaea* and *S. vulgaris* for the PA bulk suggests that adaptation may have taken place. Adaptation to PAs is known for specialized insects, which are able to sequester and use them for their own protection (Boppré, 1986). Adaptation by fungi is also known for other secondary metabolites (Morrissey and Osbourn, 1999), although additional isolates from PA-producing plants will need to be tested in order to determine the generality of fungal adaptation to PAs.

In our experiment, PAs were tested as tertiary alkaloids, while the N oxides are more common in plants. The N oxide is considered to be a nontoxic form, ideal to store in the plant or specialized insect. However, in assays studying the interaction of alkaloids with neuroreceptors and acetylcholine-related enzymes, the N oxide was sometimes more active than the tertiary alkaloid, depending on the receptor tested (Wink et al., 1998). Jain and Sharma (1987) showed that lasiocarpine did not inhibit fungal growth, but its N oxide did. In the present experiment, both the tertiary base and its N oxide were found to be inhibitory, with the N oxide slightly more effective than the tertiary base. As a consequence, the results of this

experiment with PAs in the tertiary form may underestimate the potential effects of PAs in nature.

The present results indicate that PAs may act as defense mechanisms against phytopathogenic fungi. Potential effects on saprophytic fungi, such as the *Trichoderma* sp., and beneficial fungi, such as myccorhiza, are, however, still unknown and require additional study. We still do not know the exact role of PA concentration and diversity in plants under natural conditions with respect to defense against soil borne fungi, and further research is necessary to determine the role of fungi in the evolution of PA diversity.

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GEOGRAPHIC VARIATION OF NATURAL PRODUCTS OF TROPICAL NUDIBRANCH *Asteronotus cespitosus*

SHIREEN J. FAHEY[1,2,*] and MARY J. GARSON[1]

[1]*Department of Chemistry, The University of Queensland*
Brisbane, Queensland 4072, Australia

[2]*California Academy of Sciences*
Golden Gate Park, San Francisco, California, 94118 USA

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Abstract—Extracts of the dorid nudibranch *Asteronotus cespitosus* from two geographically separate regions of Australia and from the Philippines were compared using thin-layer, high-performance liquid and gas chromatography and ^{1}H NMR analysis. Halogenated metabolites were detected in all mollusk specimens. The major component detected in digestive tissue of specimens from the Great Barrier Reef in northeastern Australia was 4,6-dibromo-2-(2′,4′-dibromophenoxy)phenol (**1**), with minor amounts of 3,5-dibromo-2-(3′,5′-dibromo-2′-methoxyphenoxy)phenol (**2**). In a specimen collected from northwestern Australia, only 3,5-dibromo-2-(3′,5′-dibromo-2′-methoxyphenoxy)phenol was found. The specimen from the Philippines contained 2,3,4,5-tetrabromo-6-(2′-bromophenoxy)phenol (**3**) together with a novel chlorinated pyrrolidone (**4**). In addition, the sesquiterpenes dehydroherbadysidolide (**5**) and spirodysin (**6**) were detected in the digestive organs and mantle tissue of the nudibranchs from the Great Barrier Reef and from the Philippines, whereas these chemicals were not found in the specimen from northwestern Australia. All of the chemicals (**1**-**3**,**5**, and **6**) have previously been isolated from the sponge *Dysidea herbacea*, as have chlorinated metabolites related to **4**. This is the first time the characteristic halogenated metabolites that typify *Dysidea herbacea* have been reported from a carnivorous mollusk, which implies a dietary origin as opposed to de novo synthesis.

Key Words—Mollusk, nudibranch, *Asteronotus*, bromophenols, sesquiterpenes, chlorinated peptides, marine chemistry, sponge chemistry, *Dysidea herbacea*, evolution, systematics.

*To whom correspondence should be addressed. E-mail: sfahey@mailbox.uq.edu.au

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INTRODUCTION

Chemical studies conducted on nudibranchs (Mollusca: Gastropoda: Opisthobranchia) have shown that their secondary metabolites (natural products) can be derived from dietary sources or from de novo biosynthesis (Cimino et al., 1985; Graziani et al., 1996; Gavagnin et al., 2001). In one of the first rigorous comparative studies on mollusk chemistry, the different sponges that had been eaten by the Pacific mollusk *Cadlina luteomarginata* were identified. The relationship between the sponge diet and the terpene metabolites detected in this mollusk was then analyzed (Hellou and Andersen, 1982; Thompson et al., 1982). Faulkner et al. (1990) subsequently reviewed the chemistry of six opisthobranch species, including *C. luteomarginata*, from the Eastern Pacific and concluded that some species show pronounced geographic variation in metabolite composition, probably due to availability of preferred food. In contrast, other species displayed the same chemistry irrespective of geographic location and were, therefore, considered likely to be synthesizing their own metabolites. In those animals that sequester chemicals, certain metabolites that were identified could vary according to geographic location (Faulkner et al., 1990; Fontana et al., 1993), or seasonally (Thompson et al., 1982), and also among animals found in the same locality (Faulkner et al., 1990). Furthermore, nudibranchs store only some of the metabolites obtained from food (Thompson et al., 1982; Cimino and Sodano, 1994; Dumdei et al., 1997a). Kubanek et al. (2000) suggested that certain species of nudibranchs might have the ability either to carry out de novo biosynthesis or to retain defensive chemicals according to habitat–specific factors or need.

To date, de novo synthesis of natural products has been detected in very few (13) species of opisthobranchs and appears to have evolved more than once, as this ability has been identified in taxonomically unrelated groups (Cimino and Ghiselin, 1998, 1999). The sources of natural products found in opisthobranchs include sponges, cnidarians, algae, tunicates, and other opisthobranchs (Avila, 1995). However, the majority of chemicals isolated thus far from nudibranchs originate from sponges upon which they feed (Cimino and Sodano, 1994; Avila, 1995; Cimino et al., 1999).

Previous workers noted that the metabolites from food may be found in the digestive tract or in the mantle tissue (Avila et al., 1990; Fontana et al., 1994, 1999) (Slattery et al., 1998), whereas metabolites synthesized de novo have a tendency to be restricted to mantle tissue or other organs used in defense (Cimino and Ghiselin, 1999; Gavagnin et al., 2001). Some studies have noted the transfer of the substance from the digestive system to organs such as the gills, the mantle dermal formations, and the cerata (Avila et al., 1990; Fontana et al., 1994, 1999; Slattery et al., 1998). It has been suggested that the tissue distribution of certain chemicals may offer evidence of de novo biosynthesis regulation (Kubanek et al., 2000).

The present study reports the possible dietary origins, anatomical distribution, and the geographic variation of the chemical metabolites of the dorid nudibranch *Asteronotus cespitosus* found in the tropical Indo-Pacific Ocean. Neither chemical studies nor reports of food sources have been previously reported in the literature for the genus *Asteronotus*, which has been placed in the family Discodoridiidae Bergh, 1891 (Valdés, in press). We report the isolation of two terpenes [spirodysin (**6**) and dehydroherbadysidolide (**5**)], three bromodiphenyl ethers [2-(2′,4′-dibromophenoxy)-4,6-dibromophenol (**1**), 2-(3′,5′-dibromo-2′-methoxyphenoxy)-3,5-dibromophenol (**2**), and 2,3,4,5-tetrabromo-6-(2′-bromophenoxy) phenol (**3**)], and a new hexachlorinated alkaloid (**4**) closely related to the sponge metabolite dysideapyrrolidone. Possible evolutionary implications of the study results are also presented.

METHODS AND MATERIALS

General Procedures. Analytical thin layer chromatography (TLC) was done using precoated silica gel Merck Kieselgel F_{254} plates with hexane–dichloromethane (1:1) as the eluant. The chromatograms were sprayed with a mixture of vanillin–sulfuric acid or with ethanolic $FeCl_3$ solution.

Gas chromatography–mass spectrometry (GC-MS) analysis was performed on a Hewlett-Packard model 5890A with an MS 5970 spectrometer. The samples were injected at 100°C using an autosampler. Dichloromethane (DCM) was used as a carrier.

High-performance liquid chromatography (HPLC) was performed on a Waters & Associates, Inc., model 501 with a Waters 410 differential detector using a μ-Porasil 300 × 7.8 mm 10 μm column, or on an Agilent 1100 with a 1100 series variable wavelength UV detector using a C-18 250 × 10.0 mm 10 μm column.

One- and two-dimensional nuclear magnetic resonance (NMR) spectra were obtained for sample solutions in $CDCl_3$, at room temperature with a Bruker DRX500 spectrometer (^{1}H, 500 MHz) with a 5-mm TXI-Zgrad probe or with a Bruker DMX750 (^{1}H, 750 MHz) with a 2.5-mm TXI-Zgrad probe.

Collection of Samples. A total of four adult specimens of *Asteronotus cespitosus* (van Hasselt, 1824) (Mollusca: Gastropoda: Opisthobranchia) were collected from three locations. Two specimens were collected from the reef flat at Heron Island, Great Barrier Reef, eastern Australia and were frozen at −70°C. One specimen was collected by SCUBA from near Enderby Island, Dampier Archipelago, northwestern Australia. This specimen was immersed in acetone at 0°C. An additional specimen was collected by SCUBA from the Batangas region in the Philippines, and stored in acetone at 0°C. All specimens were shipped to the University of Queensland, Brisbane, Australia for analysis.

Extraction and Chemical Analysis. One frozen specimen from Heron Island was dissected and the viscera, including the digestive system, was separated from the mantle tissue. The mantle tissue (approximately 30 g) was extracted with DCM–MeOH (1:1) at room temperature for 3 hr; then the solvents were removed to give a brown semisolid. The residues were partitioned in DCM, dried with $MgSO_4$, filtered through a cotton plug, and evaporated under nitrogen to yield 38 mg crude extract. This extract was purified by flash chromatography on silica using a step gradient of hexane–DCM (1:1) to 100% DCM, then 100% MeOH. Fractions containing sesquiterpenes (<1 mg) were further purified by normal-phase HPLC using 5% EtOAc in hexane to give reference samples of dehydroherbadysidolide (**5**) and spirodysin (**6**) for NMR analysis. The isolated weight of each purified terpene was approximately 0.1 mg. The recovery of each terpene could not be quantified precisely because of the volatility of these metabolites. The digestive system of the specimen was extracted separately in DCM–MeOH (1:1) at room temperature to give an olive green residue. The residue was partitioned in DCM, dried with $MgSO_4$, filtered through a cotton plug, and evaporated under nitrogen to yield 49 mg of extract. The semisolid was purified by flash chromatography on silica using a step gradient of hexane–DCM (1:1) to 100% DCM, then through 100% MeOH. Fractions containing bromophenols (1.7 mg) were further purified by normal-phase HPLC with refractive index detection and using 5% EtOAc in hexane to give 1.5 mg of 4,6-dibromo-2-(2′,4′-dibromophenoxy)phenol (**1**).

The second frozen specimen from Heron Island was dissected, and the mantle tissue (approximately 30 g) was extracted using DCM–MeOH (1:1) at room temperature, the solvent was removed to give a brown semisolid. The residue was partitioned with DCM, dried with $MgSO_4$, and the organic solution was filtered through a cotton plug and evaporated under nitrogen. The crude extract weighed 22 mg and was shown to contain dehydroherbadysidolide and spirodysin by NMR, but was not analyzed further. The digestive system of this specimen was also extracted in DCM–MeOH (1:1) at room temperature to yield 161 mg extract. The extract was purified by flash chromatography on silica using a step gradient of hexane–DCM (1:1) to 100% DCM, then through 100% MeOH. Fractions of interest were further purified by normal-phase HPLC using 5% EtOAc in hexane to give 1.7 mg of 4,6-dibromo-2-(2′,4′-dibromophenoxy)phenol (**1**) and 0.13 mg of 3,5-dibromo-2-(3′,5′-dibromo-2′-methoxyphenoxy)phenol (**2**).

For the Philippine specimen, The mantle tissue (approximately 36 g) was extracted using DCM–MeOH (1:1) at room temperature for 3 hr; then the solvents were removed to give a brown semisolid. The residues were partitioned in DCM, dried with $MgSO_4$, filtered through a cotton plug, and evaporated under nitrogen to yield 5 mg crude extract. Digestive tissue (9 g) from the specimen was extracted with DCM. The DCM-soluble portion was dried over $MgSO_4$, filtered, and evaporated under nitrogen to give 5 mg of extract. The crude extract was purified by flash chromatography on silica using a step gradient of 100% hexane, DCM,

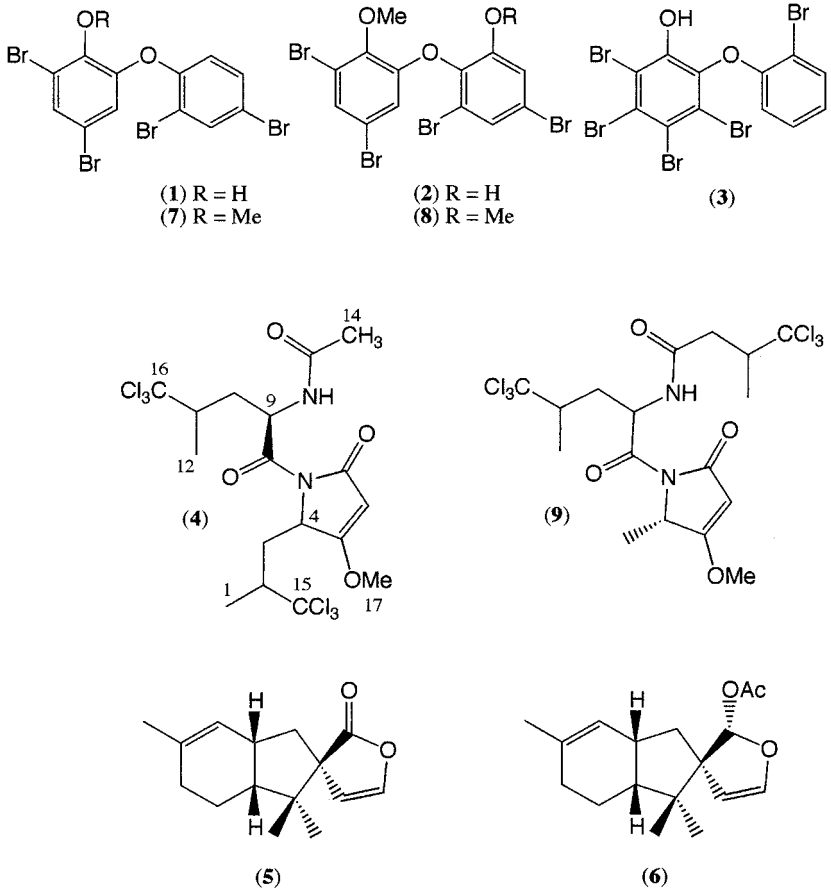


FIG. 1. Compounds found in the tropical Nudibranch *Asteronatus cespitosus*.

ethyl acetate through to 100% MeOH. The bromophenol-containing fractions were further purified by normal-phase HPLC to give <1.0 mg of 2,3,4,5-tetrabromo-6-(2′-bromophenoxy)phenol (**3**), while the chlorinated alkaloid (**4**) was isolated in 0.5 mg yield by reverse phase chromatography using MeOH–H_2O (9:1) with UV detection at 240 nm.

For the Dampier specimen, approximately half (50 ml) the acetone from the specimen collected at the Dampier Archipelago was concentrated and then partitioned with DCM. The DCM-soluble portion was dried over $MgSO_4$, filtered, and evaporated under nitrogen to give 5 mg. The crude extract was purified by flash chromatography on silica using a step gradient of 100% hexane, DCM, ethyl

acetate through to 100% MeOH. The bromophenol-containing fractions were further purified by normal-phase HPLC to give 1.4 mg of 3,5-dibromo-2-(3′,5′-dibromo-2′-methoxyphenoxy)phenol (**2**).

Methylation of Bromophenol Metabolites. Solutions of bromophenols **1** and **2** (each 1 mg) in diethyl ether were treated with diazomethane in diethyl ether at 0°C overnight; then the solvents were removed under a stream of nitrogen to give the methylated products **7** and **8** (0.8 and 0.9 mg, respectively).

Cytotoxicity Assays. Compounds **1**, **2**, **5**, and **6** isolated from *Asteronotus* were tested for cytotoxicity (IC50 correlation) against P388 cells based on luminescence detection of ATP according to standard procedures.

RESULTS

The major polybrominated diphenyl ether 2-(2′,4′-dibromophenoxy)-4,6-dibromophenol (**1**), found exclusively in the digestive tract of *A. cespitosus* collected from Heron Island, was identified by comparison of proton NMR data for the free phenol (Carté and Faulkner, 1981) and its methylated derivative (**7**) (Kuniyoshi et al., 1985; Cameron et al., 2000) with literature data. The digestive tract of both specimens from Heron Island also contained traces of a second bromophenol identified as 3,5-dibromo-2-(3′,5′-dibromo-2′-methoxyphenoxy)phenol (**2**) by comparison of the ^{1}H NMR data for the free phenol and its methylated product (**8**) with literature data (Carté and Faulkner, 1981; Norton et al., 1981). In the first specimen, the ratio of **1** to **2** was >50:1, while in the second it was 15.7:1.

Digestive tissue from a specimen collected in the Philippines contained the known 2,3,4,5-tetrabromo-6-(2′-bromophenoxy)phenol (**3**) (Salva and Faulkner, 1990) together with the novel chlorinated pyrrolidone (**4**). The structure of **4**, $[\alpha]_D + 4.8$ [$c = 0.0004$ g/ml (**4**) in $CHCl_3$)], followed from its molecular formula, determined by HR-ESMS on the (M + H) ion at 528.9796 ([^{35}Cl]$C_{17}H_{23}N_2O_4Cl_6$ requires 528.9789), together with analysis of NMR data. The ^{13}C NMR showed signals for 16 carbons only (Table 1); signals at 93.7, 168.8, and 179.0 ppm, together with a ^{1}H signal at δ 5.10 (s) were attributable to a pyrrolidone ring (Hofheinz and Oberhänsli, 1977; Unson et al., 1993). The NMR also displayed signals for two trichloromethyl groups (^{13}C; 105.3 and 105.2 ppm) and an *N*-acetate (^{1}H δg.00; ^{13}C 23.6 and 169.5 ppm). In the ^{1}H NMR, a signal at δ 2.82 (^{1}H, m, H-11) was coupled to two multiplets at δg.67 and 2.68 (H-10; each ^{1}H), and to a methyl doublet at δ 1.42 (H-12), consistent with a trichloroleucyl residue (Dumdei et al., 1997b). The δ 1.67 and 2.68 signals were further coupled to a multiplet at δ 5.77 (H-9) that was adjacent to an –*N*-H signal at δ 6.30. A second trichloroleucyl moiety could also be identified since a methyl doublet at δ 1.36 (H-1) was coupled to a signal at δ 2.77, which was coupled to multiplets at δ 2.04 and 2.40 and further coupled to a double doublet at δ 4.73 (H-4). There was an

TABLE 1. NMR DATA[a] OF PYRROLIDONE (**4**)

Position	$\delta^{13}C^b$	m	$\delta^{1}H^b$	m (J in Hz)	HMBC
1	17.3	q	1.36	d (6.5)	H-3
2	51.3	d	2.77	m (6.5)	H-1, H-3
3	35.4	t	2.40	m (14.0, 6.0)	H-1
			2.04	m (14.0, 4.0)	
4	58.2	d	4.73	dd (6.0, 4.0)	H-3, H-6
5	179.0	s	—	—	H-17
6	93.7	d	5.10	s	H-7
7	168.8	s	—	—	H-6
8	—[c]	—	—	—	—
9	51.4	d	5.77	ddd (11.0, 6.5, 6.0)	H-10
10	36.3	t	2.68	m (14.0, 6.0, 1.0)	H-12
			1.67	m (14.0, 11.0)	
11	52.2	d	2.82	m (6.5, 1.0)	H-10, H-12
12	16.8	q	1.42	d (6.5)	H-10
13	169.5	s	—	—	H-14
14	23.6	q	2.00	s	—
15	105.2	s	—	—	H-1, H-3
16	105.3	s	—	—	H-12, H-10
17	59.6	q	3.88	s	—
N-H	—	—	6.30	d (6.5)	—

[a]Bruker DRX 500 MHz; $CDCl_3$; chemical shift values referenced to $C\underline{H}Cl_3$ (δ 7.25) for ^{1}H and $C\underline{D}Cl_3$ (77.0 ppm) for ^{13}C.
[b]Assignments made from ^{1}H-^{1}H DQFCOSY, HSQC and HMBC data.
[c]Not detected in ^{13}C NMR spectrum; comparison with data for **9** suggests signal may be obscured by that of C-7 or C-13. No HMBC correlation from H-9 visible.

HMBC correlation between H-6 of the pyrrolidone ring and C-4 at 58.2 ppm. The chemical shifts of the H-10 signals are consistent with *R* stereochemistry at H-9 (Dumdei et al., 1997b; Stapleton et al., 2001); no stereochemistry could confidently be assigned to H-4. All known trichloromethyl metabolites isolated from marine sources have *S* stereochemistry at the trichloromethyl-substituted carbons (Dumdei et al., 1997b). There was an insufficient amount of (**4**) for degradative studies to confirm the proposed absolute stereochemistry.

Mantle tissue from Heron Island and Philippine samples of *A. cespitosus* did not contain these halogenated metabolites. Instead these extracts were found to contain the sesquiterpenes dehydroherbadysidolide (**5**) and spirodysin (**6**), which were identified by comparison of their GC-MS profiles with reference samples and by comparison of their ^{1}H NMR data with the literature (Kazlauskas et al., 1978; Cameron et al., 2000).

The *Asteronotus* specimen collected in northwestern Australia was stored for approximately two months in acetone and was not partitioned into mantle and digestive tissue. The specimen was shown to contain bromophenol (**2**); by

comparison with the Heron Island and Philippine data, we infer that this metabolite may have been localized in digestive tissue. Although the extract from Northwestern Australia did not contain either of terpenes **5** or **6** that were found in the Great Barrier Reef specimens, it did contain traces of other sesquiterpenes by GC-MS; however, the amounts were insufficient for analysis by 500 MHz NMR.

Compounds **1**, **2**, **5**, and **6** isolated from *Asteronotus* were tested for cytotoxicity and showed activity against P388 cells at 0.029, 0.012, 0.03 and 0.008 mg/ml, respectively. Thus, the bromophenols and terpenes showed comparable cytotoxicity, with spirodysin the most potent metabolite.

DISCUSSION

This is the first reported isolation of the two sponge terpenoid compounds, spirodysin and dehydroherbadysidolide, from a marine mollusk. In addition, three polybrominated diphenyl ethers, also found previously in sponges, are reported from a nudibranch. This is the first reported isolation of polybrominated diphenyl ethers from a carnivorous mollusk, although a closely related metabolite was found in the sea hare *Aplysia dactylomela* and in its algal diet (Kuniyoshi et al., 1985). GC-MS analysis and NMR provided the positive identification of these known compounds. A novel chlorinated pyrrolidone, structurally related to metabolites previously isolated from sponges, was also isolated. This is the first example of a marine pyrrolidone metabolite in which the heterocyclic ring may be derived from trichloroleucine and acetate (Harrigan et al., 2000).

Halogenated natural products characterize sponges of the genus *Dysidea*. Brominated diphenyl ethers have been isolated from sponges from the Great Barrier Reef (Cameron et al., 2000) and from Western Australia (Capon et al., 1981). *Dysidea herbacea* from the Great Barrier Reef is known to contain (**1**) as a secondary metabolite (Flowers and Garson, personal communication). The bromophenol (**2**) has been previously isolated from samples of *Dysidea herbacea* from Australia and the Caroline Islands (Norton et al., 1981), while (**3**) has been reported from the Philippines (Salva and Faulkner, 1990) as has dysideapyrrolidone (**9**) a close structural analogue of (**4**) (Unson et al., 1993).

According to Faulkner et al. (1990), geographic variation in the chemistry of a particular species of nudibranch indicates that the metabolites are of dietary origin rather than synthesized de novo. However, we cannot assume that the lack of variation in chemistry automatically indicates de novo synthesis because it could also simply imply that the food species are widespread.

Krug et al. (1995), noting the presence of certain chemicals in the mantle tissue of *Sclerodoris tanya*, suggested that this was an indicator of de novo synthesis if the same chemicals were absent from the digestive organs. In the case of *Asteronotus*, all the specimens examined had different chemistry. Although the

sample size is small, it appears that the chemicals vary geographically. Furthermore, the sesquiterpenes isolated were in both mantle and digestive tissue and so are unlikely to be products of de novo synthesis. Thus, we infer that the chemicals isolated from *Asteronotus* are of dietary origin. Since five out of the six metabolites are known *Dysidea herbacea* compounds, previously isolated from sponges collected in the same geographic locations as our mollusk collections, we hypothesize that *Asteronotus cespitosus* was eating *D. herbacea* or a closely related sponge. In particular, the detection of brominated and chlorinated metabolites, both of which are characteristic of *D. herbacea,* in the Philippine specimen of *Asteronotus* is suggestive of a *D. herbacea* origin, since it is highly unlikely that the nudibranch could have independently evolved two such distinctive biosynthetic capabilities. However, we have never encountered specimens of *Asteronotus* on or eating this sponge and cannot eliminate the possibility that these same chemicals may be contained in other sponge species. It appears possible, however, that *Asteronotus* has similar dietary requirements in all three geographic locations.

The results suggest that the dorid nudibranch *Asteronotus* shares traits with closely related nudibranchs. First, from our data, this species selectively sequesters certain potent chemicals, particularly sesquiterpenoids, that it probably obtains from its food source. The ability to sequester sesquiterpenes has previously been noted for other species of Doridoidea: *Cadlina* and *Tyrinna* (Cimino and Ghiselin, 1999).

Second, in the case of *Asteronotus* as well as with other dorid nudibranchs, we speculate that the animals eliminate chemicals that would be either too potent to incorporate into body tissue or not as effective as defensive chemicals. Faulkner and Ghiselin (1983) first suggested this scenario when discussing the adaptability of organisms in general. They reasoned that food materials would be selectively retained with the most defensively effective compounds accumulated, while less effective ones would be discarded. Alternatively, Pennings et al. (1999) suggested that accumulating dietary derived metabolites in the digestive organs occur to detoxify a diet that is chemically potent.

Polybrominated diphenyl ethers have been shown to be cytotoxic (this study) and active in brine shrimp lethality tests (Handayani et al., 1997). Other studies have shown these compounds to be active against gram-positive bacteria, gram-negative bacteria, and phytopathogenic fungi (Unson et al., 1993; Handayani et al., 1997), to act as enzyme inhibitors (Fu et al., 1995), and to function as potent fish-feeding deterrents (Pennings et al., 1994). Some polychlorinated metabolites show strong antifeedant properties (Unson, 1993).

Sesquiterpenes such as spirodysin are closely related in structure to terrestrial plant-derived insect antifeedants (Schulte et al., 1980). These compounds, isolated from *Dysidea herbacea* and closely related sponges, have been found to have ichthyotoxic or antifeedant properties (Thompson et al., 1982; Unson, 1993; Marin et al., 1998) as well as antifouling properties (Sera et al., 1999).

Considering the data from the present and previous studies, it seems a reasonable hypothesis that the dorid nudibranch *Asteronotus* retains the sesquiterpenes for the antifeedant and/or ichthyotoxic properties. It is possible that the brominated diphenyl ethers are eliminated as too toxic to incorporate (in view of the enzyme inhibitory properties and the results of the cytotoxicity tests performed during this study) rather than being less effective in predator deterrence. If additional live animals become available, analyses of the feces from these nudibranchs would enable us to further test our hypothesis regarding the elimination of diphenyl ethers from the dietary tract.

A third characteristic that may be shared by *Asteronotus* with closely related dorids is the additional defense mechanism of cryptic coloration that may be a consequence of diet-derived pigments. Other dorids such as *Rostanga* acquire their coloration from the sponge on which they feed (Faulkner and Ghiselin, 1983). Some *Asteronotus* have the same brownish yellow color found in some *Dysidea* sponges. It may be that *Asteronotus* developed coloration to resemble its sponge diet or its coral habitat. Although protective coloration is common in marine invertebrates, it is possible that in the case of *Asteronotus*, the camouflage may be a consequence of the sponge diet (Marin et al., 1997). If *Asteronotus* is protected chemically, crypsis may not be required for defense. Camouflage is not an effective defense against predators such as sea stars, nudibranchs, and crustaceans that do not have eyes and are not visual. However, it is possible that *Asteronotus* may be protected both chemically and visually.

Cimino and Ghiselin (1999) note that chemical defense may be the catalyst for the evolution of opisthobranch gastropods. The common ancestor of the nudibranchs is thought to have used terpenoids derived from sponges for chemical defense, and this ability is retained by *Asteronotus* (Cimino and Ghiselin, 1999). Faulkner and Ghiselin (1983) have noted that the distribution of chemicals in a phylogenetic tree will provide useful information when making evolutionary interpretations. Brunckhorst (1993) and Valdés and Gosliner (1999) used chemical metabolites as characters in phylogenetic analyses of dorid nudibranchs.

Comparison of the chemical metabolites found in *Asteronotus* with the most closely related genus, *Halgerda* (Fahey and Gosliner, 2001) will allow for evolutionary hypotheses concerning defense mechanisms. Collection and analysis of additional specimens of *Asteronotus* and other opisthobranchs, along with the sponges upon which they are found feeding can provide opportunities to examine predator–prey coevolution.

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ORIENTATION OF THE FIDDLER CRAB, *Uca cumulanta*: RESPONSES TO CHEMICAL AND VISUAL CUES

ROBERTO CHIUSSI* and HUMBERTO DIAZ

Centro de Ecologia
Instituto Venezolano de Investigaciones Cientificas
Apartado 21827, Caracas 1020A, Venezuela

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Abstract—Behavioral responses of the fiddler crab *Uca cumulanta* to flat geometric shapes mimicking natural objects were measured in a circular arena by using zonal recovery as a behavioral measurement. Crabs were tested either in presence or absence of odors from two common predator species, the blue crab *Callinectes sapidus*, and the pufferfish *Sphoeroides testudineus*. The study tested the hypothesis that *U. cumulanta* have different behavioral responses to visual cues in the presence of chemical cues associated with predators. Escape direction tests demonstrated that *U. cumulanta* is able to show zonal recovery behavior based upon astronomical references. When tested in water lacking predator odor, crabs failed to exhibit a consistent orientation if a single silhouette target was interposed in the landward direction. However, when animals were tested in different predator odor concentrations, an orientation response was obtained at 10 and 20 g/liter/hr blue crab odor and 10 g/liter/hr pufferfish odor, demonstrating *U. cumulanta* ability to detect the potential presence of its natural predators by this odor. Thus, the hypothesis was supported, and the results suggest that behavioral responses to chemical and visual cues are involved in predator avoidance.

Key Words—Fiddler crabs, *Uca*, chemical cues, vision, behavior.

INTRODUCTION

Orientation is basic to all motile animals living along shorelines (Herrnkind, 1983). Directional movement usually consists of *y*-axis orientation (Ferguson, 1967), in which animals move upshore or downshore along a line perpendicular to the shoreline. Local landmarks, beach slope, waves, and celestial cues can direct these movements. Among decapod crustaceans, *y*-axis orientation is well documented

*To whom correspondence should be addressed. E-mail: rchiussi@oikos.ivic.ve

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for brachyuran crabs (Herrnkind, 1983; Cameron and Forward, 1993), anomuran crabs (Rebach, 1981; Dunham and Schöne, 1984; Watt et al., 1985; Altizer and Forward, 1993), and shrimps (Ugolini et al., 1988; Goddard and Forward, 1989).

Visual cues, such as natural and artificial objects, including two-dimensional shapes, can influence or guide directional orientation in specific situations (Herrnkind, 1983). A shape discrimination capability in decapod crustaceans is strongly suggested by studies of visual orientation in fiddler crabs (Herrnkind, 1972, 1983; Langdon and Herrnkind, 1985), mangrove crabs (Díaz et al., 1995b), and hermit crabs (Orihuela et al., 1992; Díaz et al., 1994, 1995a).

Although visual orientation is well documented in shallow-water decapod crustaceans, there is limited knowledge about integration of information sensed by both visual and olfactory stimulation. The striped leg hermit crab, *Clibanarius vittatus*, increases the chance to encounter an empty gastropod shell by integrating proprioceptive inputs from the host shell, chemical cues, and visual recognition (Hazlett and Herrnkind, 1980; Rittschof, 1980a,b; Hazlett, 1982; Gilchrist, 1984; Rittschof et al., 1990; Orihuela et al., 1992; Díaz et al., 1994, 1995a,b). In another hermit crab, *C. antillensis*, object discrimination is based on visual and chemical information (Chiussi et al., 2001). Chemical and visual cues are also involved in predator avoidance of megalopae of the blue crab *Callinectes sapidus* (Díaz et al., 1999).

The present study examines the orientation of *Uca cumulanta* Crane, 1943 to visual and chemical cues, testing the hypothesis that adult fiddler crabs have different behavioral responses to visual cues in the presence of chemical cues associated with predators. For behavioral measurement, we utilized zonal recovery, a capability, in which, as in many other mobile intertidal animals, *U. cumulanta* is able to maintain or return—in case of previous displacement—to portions of the beach that offer them favorable conditions (Herrnkind, 1983; Pardi and Ercolini, 1986; Chiussi and Diaz, 2001). The fiddler crab *U. cumulanta* occurs only in Venezuela, Curaçao, and Suriname (Carmona-Suarez and Conde, 1996), in the intertidal zone of sheltered banks of muddy shores or sandy mud shores close to mangroves, but not under their shade. Populations are usually concentrated somewhat below mid-tide level (Crane, 1975).

METHODS AND MATERIALS

Adults of *U. cumulanta* (carapace width: 8–12 mm) were collected from the littoral zone of a small sandy shore at the perimeter of a mangrove forest (Mochima National Park, Venezuela, 10°20′N, 64°20′W). The onshore direction (YL) at the collection site was 150°, where 0° corresponds to the magnetic North. Crabs were tested at the site on the same day of collection and then returned to their habitat. They were assayed in a circular Plexiglas arena (22 cm diam.) supported on a metal

platform 1 m high. A 5-cm-high white wall surrounded the arena, with a white translucent diffuser at the bottom. The wall allowed animals to see a considerable portion of the sky, but hid the surrounding landscape from view. The bottom of the arena had a plunger attached, which was used to introduce the crabs into the center of the arena. The silhouette of a tested crab was viewed from underneath through the translucent floor. The arena was filled to a depth of approximately 4 cm with aged filtered seawater (36 ± 1 psu, $26 \pm 1^{\circ}$C). Individuals were released in seawater, because this medium provokes rapid response in fiddler crabs as well as in many other supralittoral and littoral crustaceans (Herrnkind, 1983; Pardi and Ercolini, 1986). To ensure no odors from organic sources were present in the seawater used in experiments other than those released by the experimental crabs, the seawater was filtered through an 800-kDa membrane and aged for one week in the dark.

Fifty individuals were tested in each experimental condition. A test was initiated by introducing a single animal in the center of the arena by means of the plunger. The first point of contact with the arena's wall was recorded as the orientation direction (10° accuracy). Individuals that did not reach the arena's wall within 60 sec were recorded as not responding. After each trial, individuals were placed in a large glass bowl containing aged seawater. If individuals were used in a different test on the same day, at least 30 min elapsed between trials.

Control Tests. This test was designed to verify a crab's ability to assume the onshore direction in a confined environment. Individuals were tested with astronomical cues present (e.g., sun, clear sky) at two different times of day (from 08:00 to 10:00 hr and from 12:00 to 13:00 hr). In order to test possible interpopulational changes in zonal recovery behavior due to geographical change of the habitat, a second group of 50 crabs that originated from a beach with a different YL axis (245°), was also tested.

Visual Cues. A single target attached to the outside of the arena wall served as a visual cue. It was positioned in the onshore direction. Six targets were used. They consisted of flat shapes subtending different visual angles: (1) two black horizontal rectangles (20°, 90°); (2) striped rectangles with parallel black and white bars (20°, 90°); every bar subtended a 2° angle; (3) black ovals positioned vertically and horizontally. The target dimensions were (height width, in centimeters): oval (2.48×0.87), 20° striped and black rectangles (1.90×2.48), and 90° striped and black rectangles (1.90×15.70).

Visual and Chemical Cues. Aged seawater was conditioned with odors released from an individual predator of known weight introduced in a glass container with a known volume of aged seawater and maintained for variable intervals of time to obtain different odor concentrations. For example, a predator weighting 50 g, placed in 20 liters of aged seawater for 1 hr would render an odor concentration of 2.5 g/liter/hr ($50/20 \times 1$ hr), while if the same animal is left for 2 hr,

an odor concentration of 5 g/liter/hr would be obtained. Crabs were tested for orientation to visual cues in aged seawater conditioned by two predators: the blue crab *Callinectes sapidus* Rathbun and the pufferfish (Tetradontidae) *Sphoeroides testudineus* (Linnaeus, 1758).

A preliminary predator odor trial was performed to establish the optimal predator odor concentration, i.e., the odor concentration that evoked a behavioral response to a target. In this case, we tested a horizontal oval as a target with the following odor concentrations: 2.5, 5, 10, 20, and 40 g/liter/hr. The optimal odor concentration, which was 10 g/liter/hr for both predators, was used with the six selected targets.

Laboratory Tests. Laboratory tests were carried out in the absence of astronomical references. Crabs were tested with each one of the targets with and without a chemical stimulation (predator conditioned water). In the laboratory, the illumination was provided by a circular fluorescent lamp (Philips, model TLE 32W/54) delivering an intensity of 56.5 μmol photons m^2 sec at the center of the arena. The circular lamp was positioned around the arena in a way that its outer edge was 2 cm outside the test arena wall.

Statistical Analysis. Circular statistical methods were used to analyze the data distribution (Zar, 1996). The resulting mean angle (a°) and length of the mean vector (r) were calculated for each experimental condition. The mean vector (r) has values from 0.00 to 1.00 and varies inversely with the amount of dispersion in a data set. The Rayleigh test (z) determined whether the distribution was different from uniformity. The V test for the mean angle determined whether the mean angle differed from the expected direction (YL). In the case of the visual cue tests, the expected angle was established a priori in the middle of the target. For an escape response, we predicted the expected orientation would be the opposite direction from the center of the target ($\pm 180^\circ$). The bimodality of some distributions was analyzed by doubling the angles (Zar, 1996). For the figures and tables, if the z statistic for the Rayleigh test indicated the distribution was uniform, then the distribution was designated as not significant (NS). Alternatively, if the z statistic indicated the distribution was nonuniform ($P < 0.05$), then the V test was run, and u indicated the significance level.

RESULTS

Control Tests. In all three cases by varying the relative position of the sun and YL axis, crabs oriented towards their respective onshore directions, regardless of the time of day. These results allowed us to choose 120° as the direction for the target position in all the successive tests.

Visual Cues. In water devoid of chemical cues, crabs showed no preferential response when exposed to each targets (Table 1) except for the 20° black rectangle.

TABLE 1. ORIENTATION RESPONSES OF *U. cumulanta* TO INTERPOSING DIFFERENT TARGETS TO LANDWARE DIRECTION (YL) IN DIFFERENT CONDITIONED WATER[a]

Target	*N*	*r*	*a*(°)	*z*	sig.	*u*1	sig.	*u*2	sig.	Az. (°)
No odor										
Vertical oval	48	0.240	131.93	2.765	NS					140
Horizontal oval	48	0.075	204.76	0.274	NS					142
20° black sector	46			bimodal 99.236<->279.236						165
90° black sector	50	0.082	121.22	0.340	NS					197
20° striped sector	50	0.234	268.70	2.745	NS					205
90° striped sector	48	0.205	296.42	2.025	NS					218
Pufferfish odor										
Control	42	0.403	131.85	6.828	***	3.617				120
Vertical oval	48	0.298	122.57	4.251	*	2.913	**			145
20° black sector	44	0.429	271.48	8.086	***			3.534	***	164
90° black sector	49			bimodal 42.140<-222.140						196
20° striped sector	50	0.477	262.31	11.396	***			3.778	***	205
90° striped sector	44	0.487	282.01	10.435	***			4.345	***	219
Blue crab odor										
Control	35	0.420	125.91	6.171	***	3.207	**			129
Vertical oval	46	0.251	207.19	2.903	NS					153
20° black sector	42	0.325	270.17	4.428	*			2.582	**	164
90° black sector	48	0.182	139.95	1.585	NS					196
20° striped sector	46	0.241	298.94	2.672	NS					205
90° striped sector	43	0.415	276.08	7.409	***			3.519	***	218

[a] Treatments are: no odor, pufferfish odor (10 g/liter/hr) and blue crab odor (10 g/liter/hr). Expected direction for control tests was assumed as the YL direction (150°). Symbols: *N*, responding sample size; *r*, mean vector length; *a*(°), mean vector angle, *z*, value for the Rayleigh test; sig., significance of the statistical test value (NS, not significant; $^{*}P < 0.001$ for Rayleigh test or $^{**}P < 0.005$ for *V* test); *u*1, value for *V* test calculated assuming as expected direction the center of the target (120°); *u*2, value for *V* test calculated assuming the angle opposite to the target (300°); Az., mean direction of sun azimuth during tests in the field.

In this case, the observed distribution was diametrically bimodal, towards and away from the target.

Visual and Chemical Cues. When crabs were presented with a horizontal black oval silhouette in water conditioned by different concentrations of predator odor, they exhibited an escape response with 10 g/liter/hr pufferfish odor and a diametrically bimodal distribution with 10 and 20 g/liter/hr blue crab odor (Table 2). Crabs showed no preferential response when exposed to the other concentrations of both predator species (Table 2). Therefore, we utilized concentrations of 10 g/liter/hr of both predator species with all other targets.

In the presence of 10 g/liter/hr water conditioned by pufferfish, fiddler crabs showed a significant orientation towards the vertical oval silhouette (Table 1). In contrast, they exhibited an escape response to the 20° black rectangle and the 20° and 90° striped rectangles (Table 1), orienting in the opposite direction (300°). A

TABLE 2. ORIENTATION RESPONSES OF *U. cumulanta* TO INTERPOSING BLACK HORIZONTAL OVAL TO LANDWARD DIRECTION (YL) IN DIFFERENT ODOR WATER CONCENTRATIONS[a]

Water conditioned (g/liter/hr)	*N*	*r*	*a*(°)	*z*	sig.	*u*1	sig.	*u*2	sig.	Az. (°)
Pufferfish odor										
2.5	46	0.075	1.91	0.257	NS					124
5.0	42	0.157	251.42	1.033	NS					151
10.0	46	0.298	288.55	4.082	*			2.800	**	166
20.0	47	0.238	312.05	2.673	NS					188
40.0	45	0.031	28.50	0.042	NS					210
Blue crab odor										
2.5	45	0.072	342.39	0.233	NS					133
5.0	42	0.137	246.26	0.787	NS					149
10.0	47			bimodal		113.396<->293.396				170
20.0	49			bimodal		117.656<->297.656				192
40.0	46	0.045	334.63	0.092	NS					210

[a] Fifty animals were used for each treatment.

diametrically bimodal distribution was observed when the crabs were exposed to the 90° black rectangle (Table 1).

With 10 g/liter/hr blue crab odor, animals showed an escape response away from the 20° black rectangle and the 90° striped rectangle (Table 1). When presented with the 90° black rectangle, the 20° striped rectangle and the vertical oval, the crabs oriented uniformly (Table 1).

Laboratory Tests. When surrounded by the white background with no chemical cue, *U. cumulanta* displayed a uniform distribution (Table 3), which indicated there was no directional bias due to the apparatus or technique. When presented with each one of the six targets, animals always exhibited a uniform distribution (Table 3).

In the presence of 10 g/liter/hr pufferfish odor, crabs showed significant orientation towards the 90° black rectangle (Table 3). In contrast, they exhibited an escape response to the 20° and 90° striped rectangles (Table 3). A uniform distribution was displayed with other targets (Table 3). In water with 10 g/liter/hr blue crab odor, animals showed the same distributional pattern observed in the pufferfish odor tests (Table 3).

DISCUSSION

Different stimuli can be perceived in different manners and intensities; they also can simultaneously affect animals in their natural habitat. Thus, there is a need to integrate stimuli perceived by different sensory systems (sight, smell,

TABLE 3. LABORATORY TEST OF ORIENTATION RESPONSES OF *U. cumulanta* TO DIFFERENT TARGETS IN DIFFERENT CONDITIONED WATER[a]

Target	*N*	*r*	*a*(°)	*z*	sig.	*u*1	sig.	*u*2	sig.
No odor									
Control	37	0.073	348.83	0.198	NS				
Vertical oval	45	0.013	40.50	0.007	NS				
Horizontal oval	47	0.102	6.55	0.486	NS				
20° black sector	46	0.034	191.98	0.055	NS				
90° black sector	48	0.028	258.32	0.038	NS				
20° striped sector	50	0.179	204.19	1.607	NS				
90° striped sector	49	0.153	195.14	1.150	NS				
Pufferfish odor									
Vertical oval	48	0.073	43.96	0.257	NS				
Horizontal oval	44	0.050	216.04	0.109	NS				
20° black sector	46	0.070	270.34	0.223	NS				
90° black sector	46	0.379	345.72	6.614	**	3.250	***		
20° striped sector	47	0.377	181.40	6.705	**			3.661	***
90° striped sector	49	0.462	177.24	10.463	***			4.569	***
Blue crab odor									
Vertical oval	48	0.229	33.30	0.025	NS				
Horizontal oval	46	0.055	294.21	0.138	NS				
20° black sector	46	0.098	289.29	0.442	NS				
90° black sector	42	0.330	343.13	4.585	*	2.898	**		
20° striped sector	48	0.432	184.73	8.954	***			4.217	***
90° striped sector	45	0.422	180.08	8.029	***			4.007	***

[a] Treatments are: no odor, pufferfish odor (10 g/liter/hr), and blue crab odor (10 g/liter/hr. For symbols see Table 1.

touch, etc.). Integration of stimuli results in more appropriate behavioral responses (Hazlett, 1996, 1997, 1999, 2000). The present study proves that fiddler crabs, *Uca cumulanta*, have the capability to integrate information from both visual and chemical cues. Predator odor evoked an escape response in *Uca cumulanta* when striped targets and some black targets were presented.

As observed in other decapod species (Rebach, 1981) including *Uca* species (Herrnkind, 1983), *U. cumulanta* on its home beach is able to show zonal recovery behavior based upon astronomical references, such as sun and polarized light in the sky (Chiussi and Díaz, 2001). Zonal recovery allows animals living in ecotonal environments to maintain or return to particularly favorable zones or to escape from sources of stress as quickly as possible (Herrnkind, 1983). This behavior was used as a biological measurement for evaluating the capacity of *U. cumulanta* to integrate sensory information. For this, a visual stimulus was presented alone or simultaneously with a chemical cue to test zonal recovery behavior.

When a target was presented in the predicted onshore direction of the crabs home beach (YL) in the presence of the astronomical cues, crabs failed to orient in a consistent direction, except in the presence of a 20° black rectangle (Table 1). In

the latter case, the orientation distribution was found to be symmetrically bimodal. In all other cases, crabs did not display any directional preference (Table 1). In a similar test carried out at the laboratory, without any astronomical cues, crabs also failed to orient in a particular direction (Table 3).

Chemical cues from two common predators, the pufferfish *Sphoeroides testudineus* and the blue crab *Callinectes sapidus*, were used. Since the behavioral response might vary with odor concentration (Forward et al., 1996), crab orientation was observed in tests using a single visual target (horizontal black oval) and varying odor concentrations (see Table 1). As a result, a behavioral response was obtained at blue crab odor concentration of 10 and 20 g/liter/hr and a pufferfish odor concentration of 10 g/liter/hr. The fish odor evoked an avoidance response, while the blue crab odor induced a bimodal response significantly different from a uniform distribution. Thus, the results indicate that *U. cumulanta* is able to detect the presence of its natural predators by their odor. As reported in recent studies (Mckelvey and Forward, 1995; Forward et al., 1996), chemical detection is possible within a range of concentrations, beyond which responses disappear. Using concentrations that evoke a directional orientation (Table 1), fish odor evoked an avoidance response to black oval, 20° black and striped rectangles, and 90° striped rectangle.

In contrast, when a 20° black vertical oval was presented en route to the beach, crabs oriented towards it. This response was interpreted as a refuge-seeking response. When a black rectangle subtending 90° was presented, a significant number of crabs oriented in the onshore direction but avoided the rectangle reaching the arena's wall at both ends of the target (Table 1). It seems that crabs interpret the 90° black rectangle as an obstacle in their route towards their home beach area. The same pattern, as observed in field experiments, was encountered in laboratory trials with pufferfish odor (Table 3).

The results suggest that in the presence of chemical cues *U. cumulanta* can show different responses to different shapes. There are two types of visual identification, one based on the simple dimensional characteristics of figures. In this case, a crab's response is proportional to changes in height, width, or both dimensions of the target. In the other case, identification is by association, in which an animal associates shapes to typical objects in its habitat (Langdon and Herrnkind, 1985). None of the targets presented to *U. cumulanta* resembled any object in the crab's natural surroundings. However, such targets mimic characteristics of particular objects in their habitat. Discrimination by dimensional characteristics is unlikely because *U. cumulanta* respond differently to targets of equal proportion (e.s., 90° black and striped rectangles). Similar responses have been observed for *U. pugilator* (Langdon and Herrnkind, 1985), for juveniles of the mangrove crabs *Aratus pisonii* and *Chlorodiella longimana* (Díaz et al., 1995b), and the hermit crab *Clibanarius vittatus* (Orihuela et al., 1992; Díaz et al., 1994). In these cases, the orientation responses towards the tested figures probably resulted from associating

the target dimensions to habitat elements such as marsh grass leaves for *U. pugilator*, mangrove leaves or props for *A. pisonii*, hollow spaces for *C. longimana*, and shell shapes for *C. vittatus*. In the case of *U. cumulanta*, the orientation away from the striped target might be interpreted as an escape response, with crabs viewing the striped figures as either a fish school or a seagrass meadow from which a predator might attack. Pufferfish and blue crabs inhabit seagrass meadows (Cervigon, 1978; Carmona-Suarez and Conde, 1996), and odors of both evoke escape responses (Tables 1 and 3), which supports the hypothesis that under appropriated chemical stimulation, *U. cumulanta* is able to discriminate shapes according to typical elements from its natural habitat.

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SEX ATTRACTANT PHEROMONE OF THE RED-SHOULDERED STINK BUG *Thyanta pallidovirens*: A PHEROMONE BLEND WITH MULTIPLE REDUNDANT COMPONENTS

H. L. McBRIEN,[1] J. G. MILLAR,[1,*] R. E. RICE,[2] J. S. McELFRESH,[1] E. CULLEN,[2] and F. G. ZALOM[2]

[1]*Department of Entomology*
University of California
Riverside, California 92521, USA

[2]*Department of Entomology*
University of California
Davis, California 95616, USA

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Abstract—The male-produced sex pheromone of the red-shouldered stink bug, *Thyanta pallidovirens* (Stål) (Hemiptera: Pentatomidae) consists of a blend of methyl (*E*2,*Z*4,*Z*6)-decatrienoate (*E*2,*Z*4,*Z*6–10:COOMe), and the sesquiterpenes (+)-α-curcumene, (−)-zingiberene, and (−)-β-sesquiphellandrene. In laboratory bioassays, sexually mature males attracted sexually mature females but not males, and females did not attract either sex. Extracts of volatiles collected from sexually mature males contained compounds not present in extracts from females or sexually immature males, and male-produced extract was attractive to females. Biological activity was lost when the extract was fractionated, indicating that the pheromone consisted of at least two components having different chemical properties. Individually, pheromone components were not attractive to females, but *E*2,*Z*4,*Z*6–10:COOMe in combination with at least one of the three male-produced sesquiterpenes was attractive. The presence of more than one sesquiterpene in the blend did not increase attraction, indicating redundancy in the pheromone signal. Male extract was as attractive as a blend reconstructed from synthesized compounds, indicating all biologically active components had been identified. In bioassays conducted at dusk in a 1- × 1- × 1-m screen field cage, females were attracted to synthetic pheromone lures. In field trials, adult female *T. pallidovirens* were attracted to pheromone-baited traps in relatively low numbers. The profile of volatiles released by sexually mature males of a congeneric species, *Thyanta accerra custator* McAtee, was remarkably similar to that of male *T. pallidovirens*, with the exception that

*To whom correspondence should be addressed. E-mail: jocelyn.millar@ucr.edu

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the former species produced (*E*)-2-decenal, a compound that was not found in *T. pallidovirens* extracts.

Key Words—Sex attractant pheromone, Pentatomidae, redundant, methyl (*E*2,*Z*4,*Z*6)-decatrienoate, sesquiterpene, α-curcumene, zingiberene, β-sesquiphellandrene, *Thyanta pallidovirens*, *Thyanta accerra custator*.

INTRODUCTION

True bugs in the family Pentatomidae (Heteroptera), commonly known as stink bugs, are notorious for the copious quantities of pungent defensive chemicals that they produce when disturbed (Aldrich 1988, 1995). The defensive chemicals, which are produced and stored in large metathoracic glands, are composed of blends of short-chain saturated, mono- and diunsaturated esters, aldehydes, and 4-oxo-alkenals, with the blends being qualitatively similar between species (Aldrich 1988, 1995). It is less well known that phytophagous stink bugs also produce sex pheromones from unicellular glands in the abdomen (Pavis and Malosse, 1986; Lucchi 1994). From the examples known (reviewed in McBrien and Millar, 1999), the pheromone is produced by males rather than females, and the components are chemically distinct from the defensive compounds, consisting of terpenoids, methyl esters of multiply conjugated carboxylic acids, and in one recently reported case, a macrolide lactone (Leal et al., 1998). However, the roles of the pheromones, and even the critical components of blends, have not been examined methodically and are not well understood. Studies of the pheromone blends are further complicated by the fact that stink bugs use two distinctly different sensory modalities for intersexual communication, with sex pheromones being used for longer-range orientation, and substrate-borne vibrational signals being used for intersexual communication at shorter ranges (e.g., Ota and Çokl, 1991; Çokl et al. 2001). It has also been suggested that the pheromones may stimulate production of the shorter-range vibrational signals that are used by males to locate females once they are on the same plant or substrate (Miklas et al., 1999).

Stink bugs are polyphagous and cause sporadic to chronic damage in a wide variety of crops, including grains, cotton, vegetables, berries, fruits, and nuts (Schaefer and Panizzi, 2000; McPherson and McPherson, 2000). For the past several decades, bugs often were kept to economically insignificant levels by pesticides applied to control other insects (Turnipseed and Greene, 1996). However, crop damage by stink bugs is increasing as an unintended consequence of recent fundamental changes in crop protection, such as the widespread deployment of genetically modified crop plants that express gene products from *Bacillus thurengiensis* (*B.t.*). Because the *B.t.* proteins expressed by a plant are active against only a limited number of insect families or even genera (some Lepidoptera or Coleoptera, depending on the *B.t.* strain), these plants are not protected from attack by other insects (Wilson et al., 1992; Federici, 1999). For example, the deployment of

transgenic cotton in the southeastern United States, and the subsequent reduction in pesticide applications has been correlated with increased feeding damage by pentatomid and mirid bugs (Turnipseed and Greene, 1996; Greene et al., 1999). An analogous situation has arisen in pome fruits, in which the reduction or elimination of insecticide sprays to control the primary pest (codling moth) in favor of pheromone-based mating disruption has contributed to a massive resurgence of stink bug damage in apple and pear crops (McGhee and Brunner, 1996). In a US Department of Agriculture-sponsored areawide project to control the codling moth with mating disruption, stink bug damage reached levels as high as 50% in some orchards in the Pacific Northwest in 1998 (J. F. Brunner, personal communication). In those crops, problems with stink bugs and other secondary pests may jeoparize the continued implementation of mating disruption of codling moth in the Pacific Northwest, with the threat of massive damage to fruit crops from stink bugs and other secondary pests providing an incentive for growers to resume multiple prophylactic applications of broad-spectrum pesticides.

The red-shouldered stink bug, *Thyanta pallidovirens* (Stal) (Hemiptera: Pentatomidae) is a pest of a variety of crops in western North America, including pistachios, pome fruit, tomatoes, legumes, and cruciferous crops (Rice et al., 1988; Anonymous, 1991; Schotzko and O'Keeffe, 1990). This insect is also a vector of plant pathogens, including *Botryosphaeria dothidea*, a fungal pathogen infecting pistachio (Daane et al., 2000). Because of its agricultural importance, it was chosen as a useful target for studies aimed at developing practical applications for stink bug semiochemistry. Preliminary studies of *T. pallidovirens* mating behaviors had shown that females were attracted to males, consistent with the presence of a male-produced pheromone (Wang and Millar, 1997).

Our goals in the work reported here were twofold. First, we wanted to carry out a methodical, bioassay-driven identification of the sex pheromone of the stink bug *T. pallidovirens*, with the specific aim of determining the roles of individual components in the attractant. Second, we wanted to assess whether stink bug pheromones have potential for use as attractants for sampling stink bug populations, analogous to the attractant baited traps that are used for sampling many other agricultural and quarantine pests.

We report here that sexually mature male *T. pallidovirens* produce a sex attractant pheromone with several unusual features. The pheromone is composed of four compounds, including a thermally unstable, straight-chain ester probably derived from an acetogenin biosynthetic pathway, and three chiral sesquiterpene hydrocarbons. Whereas the ester is an indispensable part of the blend, any one, any two, or all three sesquiterpenes apparently can constitute the rest of the attractant. To our knowledge, this degree of redundance is unprecedented in an insect pheromone blend. Furthermore, the profile of volatiles produced by sexually mature males of a congeneric species, *Thyanta custator accerra*, was examined, and was found to be remarkably similar to that of male *T. pallidovirens*.

METHODS AND MATERIALS

Insects. A laboratory colony of *T. pallidovirens* was started from bugs collected from alfalfa, vetch, or pistachio from sites near Davis, Fresno, and Riverside, California, USA. Field-collected individuals were added to the colony during the summer months each year. Insects were reared in a controlled environment room at 24°C ±1°C, 55 ± 10% relative humidity, with a 16L:8D photoregime provided with Sylvania Octron 32 W fluorescent lights. Insects were fed raw sunflower seeds and peanuts, organically grown green beans, and alfalfa bouquets in water. From December to April, food was supplemented with cuttings of annual weeds [London rocket, *Sisymbrium irio* (Brassicaceae) and shepherd's purse, *Capsella bursa-pastoris* (L.) (Brassicaceae)]. Food was replaced every other day.

The main colony was maintained in a wooden cage (76 × 46 × 46 cm) with filter paper (12.5 cm diam.) provided as an oviposition substrate, and new adults were added every other day. Eggs were collected from 20 breeding pairs kept individually in cylindrical paper cartons (10 × 9 cm) with screen tops; pairs were changed weekly. Eggs were collected every other day and held on filter paper in plastic Petri dishes (10 cm diam. × 2 cm high) until hatch. Newly hatched nymphs were provided with food within 24 hr of eclosion to prevent desiccation. Second-instar nymphs were transferred to larger plastic containers (16 cm diam. × 7 cm high) with two 2-cm-diam. screen-covered holes on the sides for ventilation. Fourth instars were transferred to similar, but larger plastic containers (15 cm diam. × 14.5 cm high), with two screened ventilation holes, with 20 insects per container. To increase the internal surface area and provide insect perches, a folded piece of filter paper (12.5 cm diam.) and a folded piece of 0.25-in. mesh hardware cloth were placed inside each container.

Two days after the final molt, adults for experiments (see below) were separated by sex and held as virgins in screen-topped paper cylinders (12.5 cm diam. × 16.5 cm high). After use in experiments, adults were returned to the breeding colony.

Thyanta custator accerra McAtee were field-collected from mesquite (*Prosopis* spp.) and Russian thistle (*Salsola tragus* L.) from Aricopa and Pima counties, Arizona, USA; from buckwheat *Erigonum fasciculatum* (G. Bentham) and alfalfa in Riverside County, California, and from buckwheat in San Bernadino County, California, from June to September 1998. *T. c. accerra* were reared and handled using the methods described above for *T. pallidovirens*, except food was supplemented with buckwheat.

Preparation of Insect Extracts. Headspace odors were collected from three groups of virgin adult male and female *T. pallidovirens* during August and September 1997. Headspace samples were collected from each sex separately, and the same cohort of bugs was aerated continuously for approximately 2 weeks to follow changes in the profile of compounds produced by bugs as they became sexually

mature and to collect sufficient quantities of insect-produced volatiles for analyses and bioassays. The collection apparatus was set up next to a window in a laboratory with controlled air temperature ($24 \pm 2^{\circ}$C). Natural light was supplemented with two 4-ft Sylvania Octron 32 W fluorescent lights mounted above the aeration chamber to provide a 16L:8D light cycle to prevent bugs going into diapause.

Cohorts of male and female bugs (12–24 bugs of each sex) were placed in horizontal, two-piece cylindrical glass aeration chambers (28 cm $\times$ 8.0 cm ID) with O-ring-sealed middle joints. The chambers were lined with window screen for the bugs to perch on, and three green beans and five sunflower seeds were supplied as food. Bugs were coaxed carefully into the chambers to prevent discharge of defensive secretions. Activated charcoal-filtered, humidified air (1 liter/min) was pulled through the system by vacuum. Insect-produced odors were trapped on a collector made from a glass tube (0.4 cm ID) containing a 1-cm bed of activated charcoal (50–200 mesh, precleaned by thermal desorption at 200°C under N_2; Fisher Scientific, Pittsburgh, Pennsylvania, USA) held in place by glass wool plugs. Collectors were changed every second day, at which time fresh food was also provided. Trapped odors were eluted from the collectors with 3- to 200-μl aliquots of pentane, followed by 3- to 200-μl aliquots of ether. Extracts were stored at -20°C in brown glass vials with Teflon cap liners until used for bioassays or analysis.

To determine the onset of production of male-specific odors as males became sexually mature, aeration of the first cohort began with bugs $<$39 hr old. In subsequent aerations collecting male-produced volatiles for analysis and bioassays, aerations were started with 4- to 6-day-old bugs, close to the time when pheromone production was expected to begin.

To provide materials for bioassay and analysis, a larger cohort of male bugs (77 males) was aerated in similar fashion in a larger chamber (35 cm $\times$ 15 cm ID), with an airflow of 5 liters/min and a larger collector (0.6 cm ID, 1.5-cm bed of activated charcoal).

For *T. c. accerra*, headspace volatiles were collected from seven groups of virgin adult males and three groups of females during August 1998 and from January to June 1999 (mean number of male bugs ($\pm$SEM) per cohort $= 9 \pm 2$, range $=$ 3–17 bugs per cohort; mean number of female bugs per cohort $= 7 \pm 5$, range $=$ 2–16). Aerations in August 1998 were made with field-collected insects, and later aerations used insects taken from the laboratory colony. Methods for collecting volatiles, and the equipment used, were identical to those used with *T. pallidovirens*, with the exception that we began aerations with 4- to 6-day-old bugs. Defensive compounds were not collected from *T. c. accerra*.

Collection of Defensive Compounds. Metathoracic glands (MTG) of five sexually mature virgin adults of both sexes were dissected following the method of Gilby and Waterhouse (1965), and the contents of each gland were analyzed. A drawn-out glass capillary tube was inserted into the MTG of each bug to collect the

fluid contents, the fluid was dissolved in 200 μl pentane, and the pentane solution was analyzed by gas chromatography–mass spectrometry as described below. In addition, groups of virgin adult male and female bugs were aerated separately in a glass aeration chamber for 1 hr as described above, except that the glass chamber was shaken vigorously for 3 min immediately before the aeration began, to induce discharge of defensive compounds.

Fractionation and Analysis of Insect Extracts. A solid-phase extraction cartridge (2.8 ml, containing 500 mg silica gel; Alltech #209250) was conditioned by eluting with 6 ml 25% ether in pentane, followed by 6 ml pentane. An aeration extract from mature male bugs in 2.5 ml pentane was loaded onto the cartridge, which was then eluted sequentially with 3×2 ml pentane, and 3×2 ml ether, collecting each aliquot as a separate fraction. Hydrocarbons eluted in fraction 1 (which included the pentane used to load the sample + the first 2-ml aliquot) and the ester eluted in the first ether fraction.

Extracts and fractions of extracts were analyzed by gas chromatography (GC) using a Hewlett-Packard (Avondale, Pennsylvania, USA) 5890 instrument equipped with a DB-5 (30 m $\times$ 0.25 mm ID, 25 μm film, J&W Scientific, Folsom, California, USA), a DB-17 (30 m $\times$ 0.25 mm ID, 25 μm film), or a DB-WAX (30 m $\times$ 0.32 mm ID, 25 μm film) column. Helium carrier gas was used, with a temperature program of 50°C for 1 min, then 10°C/min to 250°C for all columns. Injector and detector temperatures were 250°C and 275°C, respectively.

To confirm the identification of the thermally unstable $E2,Z4,Z6$–10:COOMe, a combined aeration extract from male bugs was analyzed by high-pressure liquid chromatography (HPLC) using a Hewlett-Packard Series 1050 instrument equipped with an Econosil column (250 $\times$ 4.6 mm, 5 μm silica; Alltech, Deerfield, Illinois, USA), eluted with 0.5% tetrahydrofuran in hexane. The eluent was monitored at 230, 254, and 300 nm with an HP 1040A diode array detector.

Identification of Pheromone Components. The identification of the male-specific ester component has been previously described (Millar, 1997). The sesquiterpene components were tentatively identified by comparison of GC retention time and mass spectral data with literature data (Adams, 1995; Joulian and König, 1998; NBS-NIH mass spectral database). Identifications were confirmed by comparisons with authentic standards.

Determination of Absolute Configurations of Sesquiterpenes. Racemic α-curcumene was resolved to baseline on a Cyclodex B capillary column (30 m $\times$ 0.25 mm ID, J&W Scientific) operated isothermally at 90°C, with 140 kPa helium headpressure. Zingiberene and β-sesquiphellandrene were not resolved on the Cyclodex B column. Consequently, zingiberene and β-sesquiphellandrene were dehydrogenated to α-curcumene (Jackman, 1960) before analysis. Thus, a 100 μl aliquot of the hydrocarbon fraction of the volatiles from males (see above) was concentrated to $\sim$10 μl in a conical vial, 100 μl of a benzene solution of 1,2,3,4-tetrachlorobenzoquinone (20 mg/ml) was added, and the vial was sealed

and heated at 80°C for 1 hr. The resulting mixture was passed through a 1-cm plug of silica gel, eluting with pentane. The filtrate was analyzed on the Cyclodex B column.

Chemicals. *E*2,*Z*4,*Z*6–10:COOMe (chemical purity >97.3%; isomeric purity >98.7%); (Millar, 1997) and (±)-α-curcumene (chemical purity >98.8%); (Hall et al., 1975) were synthesized as previously described. (−)-Zingiberene (chemical purity >98.4%; isomerically pure) was isolated from commercial ginger oil, *Zingiber officinalis* (Spectrum Chem. Co., Gardena, California, USA) as previously described (Millar, 1998). (−)-β-Sesquiphellandrene was isolated from ginger oil as follows. The oil was vacuum distilled to produce a fraction enriched in sesquiterpenes (bp 60–100 at 0.1 mm Hg). Four grams of this fraction were stirred for 48 hr with 4 g maleic anhydride in 20 ml THF to remove zingiberene as the Diels-Alder adduct. The mixture was concentrated, 100 ml hexane was added, and the mixture was stored at −20°C overnight to precipitate excess maleic anhydride. The mixture was then filtered, and the filtrate was purified by flash chromatography (5 × 25 cm column of 230–400 mesh silica gel, activated by heating at 125°C overnight), eluting with hexane (500 ml), and 500 ml each of 0.2, 0.4, 0.4, 1, and 10% CH_2Cl_2 in hexane. After combination of like fractions and Kugelrohr distillation (oven temp ~70–90°C, 0.1 mm Hg), 440 mg of impure (+)-α-curcumene (56% pure; 39% β-bisabolene), 125 mg impure β-bisabolene (77% pure), and 414 mg (−)-β-sesquiphellandrene (89.1% pure) were obtained. NMR spectra of isolated (−)-β-sesquiphellandrene were obtained on a GE QE-300 spectrometer: ^{1}H NMR (300 MHz): δ 6.16 (dd, 1H, J = 9.9, 2.5 Hz), 5.70 (d, 1H, J = 9.7 Hz), 5.12 (m, 1H), 4.75 (br s, 2H), 2.44 (dt, 1H, J = 16.5, 4.6 Hz), 2.3 (m, 1H), 2.23 (m, 1H), 2.0 (m, 2H), 1.74 (m, 1H), 1.70 (s, 3H), 1.62 (s, 3H), 1.57 (m, 1H), 1.4 (m, 2H), 1.22 (m, 1H), 0.86 (d, 3H, J = 8 Hz). ^{13}C NMR (75.48 MHz): δ 15.9, 17.7, 24.4, 25.7, 26.0, 30.4, 34.3, 36.6, 40.5, 109.8, 124.7, 129.5, 131.2, 135.2, 143.8. The carbon and proton spectra were a satisfactory match to literature data (Sy and Brown, 1997).

Y-Tube Bioassays. Two-choice bioassays with a vertical glass Y-tube were carried out with virgin adult males and females (9–12 days old) as previously described (McBrien et al., 2001).

Live Bugs as a Pheromone Source. All four possible combinations of potential pheromone producers (with a green bean) and responders were bioassayed (attraction of females to odors from males on a bean, females to females, males to females, and males to males) versus a control (a green bean in a flask). All insects used as sources of attractant and as responders were sexually mature virgins. Because females were attracted to males, and all other combinations failed to demonstrate attraction, females were used as responders in all further experiments.

Insect Extracts as a Pheromone Source. Ten bug-hour equivalents of crude male extract in pentane (containing approximately 2 μg *E*2,*Z*4,*Z*6–10:COOMe, 4 μg α-curcumene, 4 μg zingiberene, and 0.4 μg β-sesquiphellandrene) were

bioassayed versus a pentane control, with virgin female *T. pallidovirens* responders. Extracts or solvent controls were pipetted onto circular pieces of filter paper (1.5 cm diam.) held in the top of each arm by wire loops inserted inside the top joints of the Y tube.

A portion of the male extract was fractionated into an ether and a pentane fraction (see above); the major component of the ether fraction was *E*2, *Z*4, *Z*6–10:COOMe, and the major components of the pentane fraction were three sesquiterpenes, α-curcumene, zingiberene, and β-sesquiphellandrene. The biological activity of each fraction was tested against a pentane blank, with the amounts of each fraction standardized so that each component was present in the same amounts as in the whole extract.

Synthetic Chemicals as a Pheromone Source. The amounts of each pheromone component (*E*2,*Z*4,*Z*6–10:COOMe, ($\pm$)-α-curcumene, ($-$)-zingiberene, and ($-$)-β-sesquiphellandrene) used in laboratory bioassays were equivalent to the amounts present in attractive male extract (see above), except that the amount of α-curcumene was doubled because the synthetic compound was racemic (2 μg of ester, 8 μg of α-curcumene, 4 μg of zingiberene, and 0.4 μg of β-sesquiphellandrene). The following treatments were tested versus pentane controls: (1) each individual component; (2) ester plus one sesquiterpene, for all possible binary blends; (3) ester plus two sesquiterpenes, all blends; and (4) complete four-component blend. The complete blend was also tested versus each of the two- and three-component blends in parts 2 and 3 above. Finally, the two-component blend of the ester plus ($-$)-β-sesquiphellandrene was assayed versus male extract. The previous bioassays had shown that this blend was as attractive as all other combinations of ester and sesquiterpene, so it was used for testing against the extract. The amounts of each component in the insect extract and the reconstructed blend were equal.

Field Cage Study Using Synthetic Pheromone Components. ($\pm$)-α-Curcumene and *E*2,*Z*4,*Z*6–10:COOMe were synthesized as described above. Zingiberene was isolated from ginger oil, and ($-$)-β-sesquiphellandrene was synthesized as described by Chavan et al. (1997), as an $\sim$1:1 mixture with its C-6 epimer ($>$96.8% of the purified product). Because the synthesized material was a 1:1 mix of epimers, the dose of the synthetic mixture used on lures was doubled.

A 1- $\times$ 1- $\times$ 1-m field cage was set up in the Agricultural Operations experimental fields, University of California, Riverside, following the protocols described by McBrien et al. (2001). Fifteen minutes before the start of each bioassay, two freshly cut bouquets of alfalfa were positioned 0.5 m apart on the cage floor, and a 1-m piece of cotton yarn was strung from the base of each bouquet to a wire loop attached to the roof immediately above each bouquet. At the top of each string, the base of a 4-cm-long stalk of alfalfa was attached to the roof using a wire loop and positioned so that it extended straight down beside the string. Several of the insects walked on the roof of the cage during the trial and could approach the strings from the top instead of the cage floor. The treatment string lure was saturated with 3.5 ml

of pentane containing 0.4 mg of $E2,Z4,Z6$–10:COOMe, 1.6 mg of α-curcumene, 0.8 mg of zingiberene, and 0.16 mg of β-sequiphellandrene, and the control string was soaked with 3.5 ml of pentane alone. The pentane was allowed to evaporate, and the strings were hung from the cage roof, with the position of the treatment and control strings alternated for each replicate.

Thirty minutes before beginning a bioassay, 13–32 virgin female *T. pallidovirens* (mean $\pm$ SD:21 $\pm$ 8.5), were placed in a 25- $\times$ 15- $\times$ 15-cm cardboard box with two green beans. The inside walls of the box were lined with Fluon-coated sticky tape to prevent insects from crawling out of the box, the bottom of the box was lined with plain white paper that could be lifted out (see below), and the top was covered with fine mesh cloth. After 10 min, the beans were removed, and the insects were introduced into the field cage (2–3 min after the strings were positioned), being careful to minimize disturbance of the bugs. The mesh screening was removed from the box and placed just inside the front wall of the field cage so that any insects on the screening would be free to disperse in the cage. The paper liner was lifted out of the bottom of the box and placed inside the front wall beside the screening, equidistant from each bouquet. The bioassay was repeated on six evenings during August 1999, approximately 40 min before sunset. After 40 min, the number of females on each bouquet was counted.

Field Trials. Three field trials were carried out in tomato fields near Davis, California, USA, from July to September 1998 and during September 1999 using commercial stink bug traps (Sierra Ag., Fresno, California, USA) modeled after those described by Aldrich et al. (1991). Traps were placed beside tomato plants so that they were touching the vegetation and insects could crawl into the screen-cone openings. Treatments within a replicate were randomly assigned to each trap, and traps were placed at least 10 m apart. Treatments were replicated three to six times, and once every 7 days, insects in each trap were removed, identified to species and sex, and counted. Baits consisted of 11-mm red rubber septa (Wheaton, Millville, New Jersey, USA) loaded with methylene chloride or hexane solutions of the test compounds and with 0.5% butylated hydroxytoluene (BHT) added as stabilizer.

In an attempt to achieve approximately the same profiles of compounds from a rubber septum bait as were seen in the GC traces of male-produced bug volatiles, the amount of each compound loaded onto a septum for placement in the field was adjusted for differences in volatility. These differences were estimated using the following procedure. Red rubber septa ($N = 3$) were loaded with 2 mg of methyl ($E2,Z4$)-decadienoate (a thermally stable ester with one less double bond than $E2,Z4,Z6$–10:COOMe; Bedoukian Research, Danbury, Connecticut, USA), 5 mg of the sesquiterpene hydrocarbon fraction of ginger oil, and 0.75 mg of BHT in 100 μl of methylene chloride, and left to dry for 3 hr. Septa were then placed in the glass aeration chambers used to collect volatiles from bugs (see above). Air purified by passage through an activated charcoal filter was pulled through the system by vacuum at a flow rate of 1 liter/min. The headspace volatiles were trapped

on a 1-cm bed of activated charcoal held in a glass tube (0.4 cm ID) as described above. The relative amounts of the four compounds in the original solution before loading on septa were compared to the relative amounts emitted by septa over time. Collectors were changed hourly for the first 3 hr, and then once a day for 7 days. Collectors were eluted as described above, and the relative amounts of zingiberene, α-curcumene, β-sesquiphellandrene, and methyl ($E2,Z4$)-decadienoate in extracts were determined by GC analysis, for each collection period. Because the ratio of the four components was fairly constant from 3 days onward, the mean values for days 3–7 were calculated and compared to the original solution. Using β-sesquiphellandrene as the standard, the ratios of ester, α-curcumene, and zingiberene to β-sesquiphellandrene were 1.5, 2.0, and 1.3 times higher in aeration extracts than in the original solution. These correction factors were used when preparing lures for the field.

The first field test (July 22–August 12, 1998) compared the attraction of bugs to traps baited with red rubber septa treated with hexane solutions of $E2,Z4,Z6$–10:COOMe and the sesquiterpene hydrocarbon fraction of ginger oil [see above; 69% (+)-α-curcumene, (−)-zingiberene, and (−)-β-sesquiphellandrene in a ratio of 0.62:2.29:1, plus 31% other sesquiterpenes] to traps baited with solvent-treated controls. Each lure was loaded with 10 mg ester, 29 mg oil, and 1.67 mg BHT, and treatments were replicated six times.

A second field trial (August 21–September 4, 1998), replicated three times, compared the four-component blend of $E2,Z4,Z6$–10:COOMe + the three sesquiterpenes, binary blends of $E2,Z4,Z6$–10:COOMe and one sesquiterpene, ester alone, and a solvent-treated control. Lures were loaded with 5 mg of $E2,Z4,Z6$–10:COOMe plus appropriate amounts of the sesquiterpenes, using the purified materials used in the laboratory bioassays.

A final field trial (September 1–15, 1999) retested the same treatments (four replicates) as used in the second experiment, with the inclusion of a treatment consisting of the mixture of the three sesquiterpenes alone, with no ester.

Statistical Analysis. Y-tube bioassay data were analyzed using a χ^2 test with one degree of freedom, using the Yates correction factor (Steel et al., 1997). Because different numbers of bugs were used for each replicate in the field-cage studies, the proportion of bugs on each bouquet, as a percentage of the total number of bugs on both bouquets, was calculated for each replicate. After arcsine transformation (Steel et al., 1997), means were compared with a two-sample t test.

Because of the low trap catches in field trials, trap catches for each treatment and sex were pooled for the duration of each experiment. In the first field trial, data were analyzed using a nonparametric two-factor analysis of variance, with treatment and sex as the main factors (Zar, 1984). In all statistical tests, $\alpha = 0.05$.

Trap catches per treatment in trials 2 and 3 were too low for statistical analysis. However, the results are presented because they corroborate the results of the laboratory and field cage bioassays.

RESULTS

In Y-tube bioassays, odors from sexually mature males attracted female bugs [treatment 25, control 5, $\chi^2 = 12.03$, $P < 0.001$), but not males [treatment 13, control 11, $\chi^2 = 0.25$, not significant (NS)], and live females did not attract either sex (female responders: treatment 10, control 10; male responders, treatment 12, control 8, $\chi^2 = 0.017$, NS). Based on these results, further bioassays tested only the attraction of sexually mature females to a pheromone source.

An extract of male-produced volatiles collected on activated charcoal was more attractive to female *T. pallidovirens* than nontreated controls (extract 27, control 3; $\chi^2 = 17.63$, $P < 0.001$). Comparisons of gas chromatograms of aeration extracts from sexually immature and mature males, and sexually mature males and females (Figure 1) determined that sexually mature male *T. pallidovirens* produced at least four compounds that were not present in females or immature males. Furthermore, the initiation of pheromone production in males coincided with the onset of sexual maturity as determined by Wang and Millar (1997).

Fractions of the extract eluted from silica gel with pentane and ether were not attractive (pentane 11, control 13, $\chi^2 = 0.042$, NS; ether 8, control 12, $\chi^2 = 0.45$, NS), suggesting that the attractant was a synergistic blend of at least two

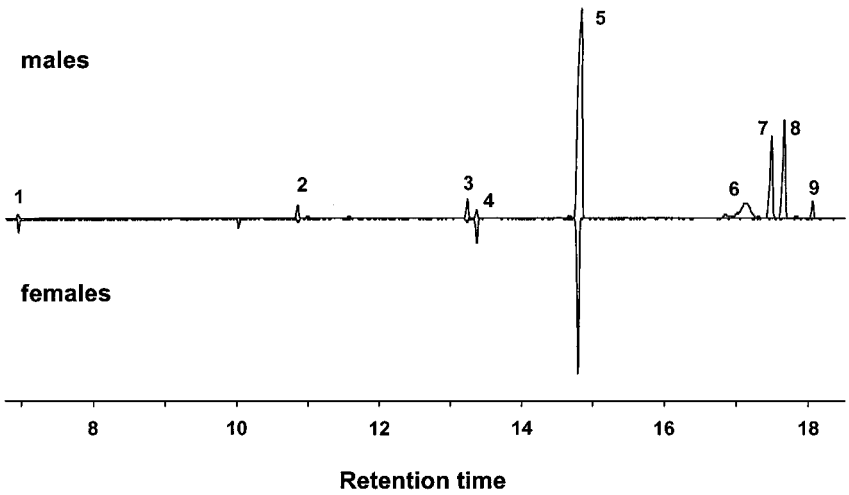


FIG. 1. Gas chromatograms of extracts of volatiles collected from male (top) and female (bottom, inverted) *Thyanta pallidovirens*. 1, (*E*)-2-hexenal; 2, (*E*)-2-octenal; 3, dodecane; 4, (*E*)-2-octenyl acetate; 5, tridecane; 6, distorted peak from thermal decomposition of methyl *E*2,*Z*4,*Z*6-decadienoate; 7, (α)-curcumene; 8, zingiberene; 9, β-sesquiphellandrene. Retention time is in minutes.

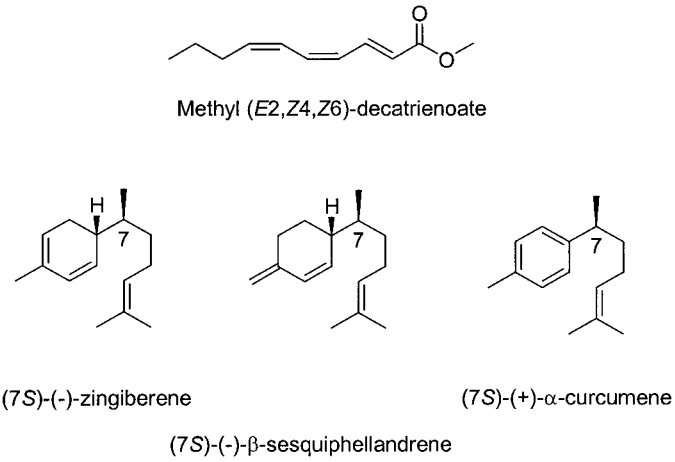


FIG. 2. Structures of male-specific compounds produced by adult *Thyanta pallidovirens*.

components with different polarities. Analysis of the two fractions by GC and GC-MS determined that the ether fraction consisted exclusively of $E2,Z4,Z6$–10:COOMe (Millar, 1997) (Figure 2). The pentane fraction contained three male-specific sesquiterpene hydrocarbons, all with bisabolane skeletons (Figure 2). The first two of these were tentatively identified from their distinctive mass spectra and matches with database spectra (NBS-NIH database) as α-curcumene [m/z (abundance) 202 (34), 119 (100), 132 (86)] and zingiberene [m/z 204 (13), 119 (100), 93 (99)]. The third compound [m/z 204 (15), 161 (31), 133 (26), 120 (23), 93 (53), 92 (33), 91 (47), 77 (42), 69 (100), 55 (33), 41 (99)] was tentatively identified from a combination of literature retention indices and mass spectra (Adams, 1995; Joulain and König, 1998) as β-sesquiphellandrene, an isomer of zingiberene. The identifications were confirmed by matching mass spectra and matching retention times on DB-5, DB-17, and Cyclodex B GC columns (see below) with those of authentic standards. The mean ratio of α-curcumene to zingiberene to β-sesquiphellandrene in extracts from three cohorts of male bugs (bugs per cohort = 16, 77, and 11, respectively) was 9.4:9.8:1.0 (range of values versus β-sesquiphellandrene: for α-curcumene, 13.3–6.5; zingiberene, 10.4–9.2). Because the ester was destroyed during GC analysis, it was not possible to determine the ratio of the ester to the sesquiterpenes accurately by GC.

The absolute configurations of the three sesquiterpenes were determined as follows. First, racemic α-curcumene was resolved to baseline on a Cyclodex B GC column (90°C isothermal, retention times 82.08 and 83.38 min), and the earlier eluting peak was identified as the (7*S*)-(+)-enantiomer (König et al., 1994; Joulain and König, 1998) by injection of an authentic sample isolated from ginger oil.

Although the retention times of the insect-produced zingiberene and β-sesquiphellandrene matched those of standards of (7*S*)-(−)-zingiberene and (7*S*)-(−)-β-sesquiphellandrene isolated from ginger oil (Buckingham, 1994; König et al., 1994), samples of the opposite enantiomers and the racemic forms of these two compounds were not available. Consequently, another method of conclusively proving the absolute configurations was required. Thus, the sesquiterpene fraction from the male volatiles was dehydrogenated with 3,4,5,6-tetrachloro-1,2-benzoquinone (Jackman, 1960), converting the insect-produced zingiberene and β-sesquiphellandrene to α-curcumene. The resulting sample gave a single peak on the Cyclodex B column at the retention time of (7*S*)-(+)-α-curcumene, with no trace of the 7*R* enantiomer, proving that the configuration at the 7 position in the insect-produced α-curcumene, zingiberene, and β-sesquiphellandrene must be *S* for all three compounds.

A number of other compounds were identified in the aeration extracts from both sexes, including (*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-2-octenyl acetate, undecane, dodecane, and tridecane. These compounds, and others, were found in the metathoracic gland contents, and most were also found in aeration extracts from bugs that had been disturbed by vigorous shaking (Table 1). These compounds were tentatively identified by comparison of spectra with those in the NIH-NBS mass spectral database, and identifications were confirmed by comparison of GC

TABLE 1. COMPOUNDS IN METATHORACIC GLANDS OF FIVE MALE AND FIVE FEMALE *Thyanta pallidovirens* RELATIVE TO MOST ABUNDANT COMPOUND, TRIDECANE[a]

	Amount (%, mean ± SE)			
	Metathoracic glands		Shaken bugs	
Compound	Males	Females	Males	Females
(*E*)-2-Hexenal	7.3 ± 2.1	7.5 ± 1.1	3.7	
(*E*)-2-4-Oxo-hexenal	9.3 ± 2.9	7.1 ± 3.5		
(*E*)-2-Hexenyl acetate	1.2 ± 0.6	0.8 ± 0.4		
(*E*)-2-Octenal	13.2 ± 4.2	9.1 ± 2.7	6.3	0.07
(*E*)-2-Octenol	0.04 ± 0.02	0.2 ± 0.1		
Undecane	0.5 ± 0.1	0.4 ± 0.08	0.7	0.05
Dodecane	3.4 ± 0.5	3.7 ± 0.5	3.2	0.3
(*E*)-2-Octenyl acetate	9.6 ± 3.9	10.5 ± 3.8	1.7	0.004
(*E*)-2-Decenal	0.002 ± 0.002	0		
Tridecane	100	100	100	100
Tetradecane	0.0700 ± 0.01	0.06 ± 0.007		
(*E*)-2-Decenyl acetate	0.006 ± 0.006	0.01 ± 0.01		
Pentadecane	0.3 ± 0.04	0.3 ± 0.04		

[a]Also shown are mean percentages of compounds (relative to tridecane) in extracts from bugs disturbed by shaking.

retention times (DB-5 and DB-17 columns) and mass spectra with those of authentic standards. (*E*)-2-4-oxo-hexenal was also present in extracts from both sexes and was tentatively identified by comparison with a reference spectrum from J. R. Aldrich (US Department of Agriculture, Beltsville Maryland, USA). The identification was confirmed with a synthesized standard (Marques et al., 2000). These compounds are well-known stink bug defensive chemicals (Aldrich, 1995).

Having identified the male-specific compounds in the aeration extracts and synthesized or isolated quantities sufficient for testing, laboratory bioassays were continued with *E*2,*Z*4,*Z*6–10:COOMe, (±)-α-curcumene, (−)-zingiberene, and (−)-β-sesquiphellandrene. None of the synthetic pheromone components alone were attractive when compared to pentane controls, but binary blends of *E*2,*Z*4, *Z*6–10:COOMe with any one of the three sesquiterpenes were significantly more attractive than controls (Table 2), demonstrating that the pheromone blend consists of at least two components. When given a choice between the four-component (4-C) blend of *E*2,*Z*4,*Z*6–10:COOMe plus all three sesquiterpenes and a pentane blank in the Y-tube bioassay, the 4-C blend was more attractive to female *T. pallidovirens*(treatment, 28, control 2 responders; $\chi^2 = 20.83$, $P < 0.001$). However, further bioassays demonstrated that the 4-C blend was no more attractive than any blend of *E*2,*Z*4,*Z*6–10:COOMe with any one or any two of the sesquiterpenes (Table 3). To our knowledge, this degree of redundance is unprecedented in an insect pheromone blend.

In a final Y-tube bioassay, there was no difference in the responses of females to a binary blend of *E*2,*Z*4,*Z*6–10:COOMe plus (−)-β-sesquiphellandrene and an extract of volatiles from males (synthetic blend 14, extract 11 responders; $\chi^2 = 0.16$, $P > 0.05$), indicating that the two-component blend was as attractive as the insect-produced volatiles.

TABLE 2. RESPONSES OF FEMALE *Thyanta pallidovirens* TO INDIVIDUAL PHEROMONE COMPONENTS OR BLENDS OF PHEROMONE COMPONENTS COMPARED TO PENTANE CONTROLS IN Y-TUBE BIOASSAY[a]

Treatment	Number of responders		χ^2
	Treatment	Control	
*E*2,*Z*4,*Z*6–10:COOMe	14	17	0.13 ns
(±)-α-Curcumene	5	15	4.05*
(−)-Zingiberene	8	12	0.45 ns
(−)-β-Sesquiphellandrene	10	10	0.05 ns
*E*2,*Z*4,*Z*6–10:COOMe + (±)-α-curcumene	25	5	12.03***
*E*2,*Z*4,*Z*6–10:COOMe + (−)-zingiberene	24	6	9.63**
*E*2,*Z*4,*Z*6–10:COOMe + (−)-β-sesquiphellandrene	26	4	14.70***

[a] χ^2 values followed by asterisks indicate significant differences between treatments and controls (*$P < 0.05$; **$P < 0.005$; ***$P < 0.001$).

TABLE 3. FEMALE *Thyanta pallidovirens* RESPONDING TO 4-COMPONENT (4-C) BLEND[a] vs. BLENDS OF *E*2,*Z*4,*Z*6–10:COOMe WITH TWO SESQUITERPENES OR ONE SESQUITERPENE IN Y-TUBE BIOASSAY[b]

Treatment	Number of responders		χ^2
	Treatment	4-C blend	
4-C blend–(−)-zingiberene	10	10	0.05
4-C blend–(±)-curcumene	9	11	0.05
4-C blend–(−)-β-sesquiphellandrene	10	10	0.05
*E*2,*Z*4,*Z*6–10:COOMe + (−)-β-sesquiphellandrene	15	11	0.35
*E*2,*Z*4,*Z*6–10:COOMe + (±)-α-curcumene	11	9	0.05
*E*2,*Z*4,*Z*6–10:COOMe + (−)-zingiberene	11	11	0.05

[a] 4-C blend: (*E*2,*Z*4,*Z*6–10:COOMe, (±)-α-Curcumene, (−)-zingiberene, and (−)-β-sesquiphellandrene)
[b] No treatment was significantly different from the 4-C blend by χ^2 analysis.

Field Cage Study. The four-component synthetic blend was more attractive to females than a pentane control in field cage trials (treatment, mean ± SE, 77.8 ± 1.9% of responding bugs; control 22.2 ± 1.9%, $t = 6.19$, $df = 10$, $P < 0.001$). During these trials, most of the movement towards the baited alfalfa bouquets occurred within the first 10–15 min after the bugs were introduced into the cage. Any flight occurred immediately after introduction of insects into the cage, but bugs almost never flew directly to the alfalfa bouquets, only to the cage walls or ceiling. Females approached baited alfalfa bouquets almost exclusively by walking or a series of short hops. Once on the alfalfa bouquets, all but three of the bugs remained there for the duration of the trials. Of all the bugs released, an average of 48.4 ± 2.3% had not gone to either bouquet by the end of the trial and were classified as nonresponders. Most of these bugs wandered around the roof or floor of the cage and aggregated around the perimeter of the cage where the roof and walls joined.

Field Trials. Although trap catches were low in all field tests, reconstructed blends of the male-produced compounds were attractive specifically to adult female *T. pallidovirens*. In the first trial, 19 female and 1 male *T. pallidovirens* were caught in traps baited with synthetic pheromone, which was more than the single female *T. pallidovirens* trapped in nonbaited controls (nonparametric two-way ANOVA, $\chi^2_{0.05,1} = 5.46$, $P < 0.025$). A total of 60 *T. pallidovirens* females and 3 males were captured in trials 2 and 3 (Table 4). Bugs were caught only in traps baited with blends of *E*2,*Z*4,*Z*6–10:COOMe with at least one sesquiterpene; no bugs were caught in traps baited with the ester alone, the sesquiterpene blend, or the control, and no nymphs were caught in any trap.

Traps baited with *T. pallidovirens* pheromone components were also species-specific. The only other stink bug species found in traps was *Euschistus conspersus*.

TABLE 4. *Thyanta pallidovirens* FEMALE AND MALE ADULTS CAPTURED IN JUG TRAPS BAITED WITH SYNTHETIC SEX PHEROMONE COMPONENTS OR SOLVENT-TREATED CONTROLS[a]

	Lure composition							
Responder	e,s,c,z	e + s	e + c	e + z	e	s,c,z	Blank	Total
Trial 2:								
Female	6	3	4	17	0	[b]	0	30
Male	0	0	0	1	0	[b]	0	1
Trial 3:								
Female	5	7	6	12	0	0	0	30
Male	0	0	1	1	0	0	0	2

[a] Traps were placed in tomato fields near Davis, California, USA. Trial 2: August 21–September 4, 1998; 3 replicates. Trial 3: September 1–15, 1999; 4 replicates. Lure composition codes: e = ester (*E*2,*Z*4,*Z*6–10:COOMe), s = (−)-β-sesquiphellandrene, c = (±)-α-curcumene, z = (−)-zingiberene.
[b] Trial 2 did not contain this treatment.

During trials 1 and 3, respectively, one *E. conspersus* female, and two *E. conspersus* females and one nymph were caught in traps baited with *T. pallidovirens* pheromones. However, during trials 1 and 2, traps baited with *E. conspersus* pheromone captured 98 and 47 adult *E. conspersus*, respectively, in the same fields during the same period, indicating that *E. conspersus* populations were present and that the few individuals caught in the *T. pallidovirens* traps were probably random captures.

Analysis of Volatiles from Thyanta custator accerra. The profile of volatiles produced by sexually mature male *T. c. accerra* ($\geq$10 days old) was qualitatively and quantitatively very similar to that of male *T. pallidovirens* (Figure 3). The extracts differed only in the presence of (*E*)-2-decenal in the *T. c. accerra* extracts (Figure 3), the structure of which was confirmed by retention time and mass spectral matches with a synthetic standard. *E*2,*Z*4,*Z*6–10:COOMe was found in all aeration extracts from sexually mature males, and HPLC analysis, as described above for *T. pallidovirens* extracts, proved that the insects produced only the *E*2,*Z*4,*Z*6 isomer. The mean proportions of the three sesquiterpenes and (*E*)-2-decenal, with β-sesquiphellandrene arbitrarily set at 1, were α-curcumene, 10.5 ± 0.8; zingiberene, 15.4 ± 1.1; and (*E*)-2-decenal, 3.1 ± 0.6. The absolute configurations of the sesquiterpenes were not determined. The ester, the three sesquiterpenes, and (*E*)-2-decenal were not found in extracts from sexually immature males, sexually immature females, or sexually mature females.

DISCUSSION

Only adult female *T. pallidovirens* were attracted to insect-produced or synthetic pheromone blends, indicating that the male-produced compounds constitute

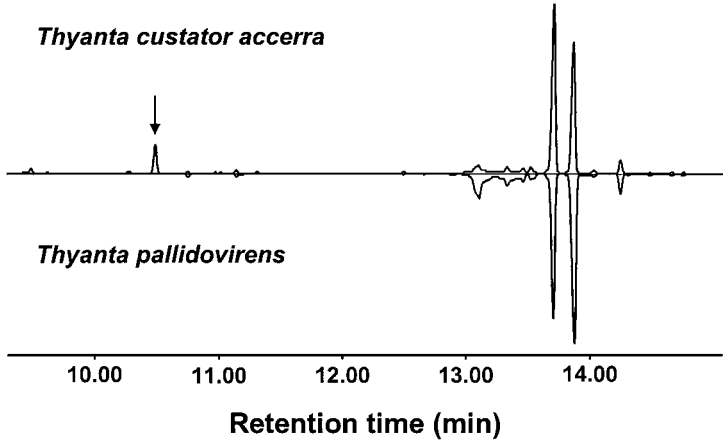


FIG. 3. Gas chromatograms of extracts of volatiles collected from sexually mature male *Thyanta custator accerra* (top trace) and *Thyanta pallidovirens* (bottom trace, inverted). The arrow indicates the presence of (*E*)-2-decenal in the *T. c. accerra* extract. The other peaks are as labeled in Figure 1.

a sex pheromone rather than an aggregation pheromone, as is the case with the stink bug *Acrosternum hilare* (McBrien et al., 2001). In contrast, males of several other phytophagous pentatomids are reported to produce aggregation pheromones that attract both sexes and in some cases nymphs (Harris and Todd, 1980; Aldrich et al., 1991; James et al., 1996; Sugie et al., 1996; Leal et al., 1998). However, it must be pointed out that these apparent aggregations may be the result of stimuli other than the pheromone being tested, such as chemical attractants or arrestants, or vibrational signals produced by bugs already caught in the traps.

There are several possible explanations for the production of attractant pheromones by male rather than female bugs. For example, male *T. pallidovirens* invest heavily in reproduction, with virgin males donating 17% of their body mass to virgin females during mating (Wang and Millar, 1997). There is also evidence of male mate choice in *T. pallidovirens*, with males terminating copulation with small females before sperm was transferred (Wang and Millar, 1997). The important role that males play in attraction, courtship, and assessment of females may contribute to the adaptive significance of the attractant pheromone being produced by males.

Selective pressure from natural enemies may offer an alternative explanation for males being the pheromone-producing sex. Stink bugs suffer heavy predation from specialist predators and parasitoids that eavesdrop on the bugs' semiochemical emissions to locate their prey (Mitchell and Mau, 1971; Aldrich et al., 1989; Aldrich, 1995; Millar et al., 2001). Because female *T. pallidovirens* lay a number of egg masses spaced out over their adult lifetime (Wang and Millar, 1997), their reproductive potential is directly related to their longevity. In contrast, a male bug

mating with a virgin female transfers enough sperm to fertilize 50% of the eggs that she produces during her lifetime (Wang and Millar, 1997). Consequently, the risks of signaling, although still high, may be less for males than females.

Of the four sex-specific compounds produced by *T. pallidovirens* males, *E*2,*Z*4,*Z*6-10:COOMe is a novel natural product. However, the *E*2,*E*4,*Z*6–10:COOMe isomer recently has been identified as a male-produced pheromone component of another pentatomid, the brown-winged green bug, *Plauti stali* Scott (Sugie et al., 1996). Both of these esters also are closely related to *E*2,*Z*4–10:COOMe, a pheromone component of several *Euschistus* spp. (Aldrich et al., 1991). Thus, the unsaturated straight-chain 10-carbon methyl ester is a recurring motif in stink bug semiochemistry.

The three male-produced sesquiterpenes, (+)-α-curcumene, (−)-zingiberene, and (−)-β-sesquiphellandrene, are relatively common components in essential oils, and in a curious coincidence, the major portion of the sesquiterpene fraction of commercial ginger oil consists of these three compounds, even to the extent of having the same stereochemistry. β-Sesquiphellandrene [possibly the (−) enantiomer], was recently identified as a pheromone component of the pentatomid *Piezodorous hyberni* (Leal et al., 1998), but neither α-curcumene nor zingiberene have been reported previously from heteropteran secretions.

During our studies with *T. pallidovirens*, we had an opportunity to examine the volatiles of a congener, *T. custator accerra*, which is sympatric with *T. pallidovirens* in the southwestern United States. Morphologically, these species are difficult to differentiate (McPherson and McPherson, 2000) but there can be no doubt that they are different species because they have different chromosome numbers (Rider and Chapin, 1992). Although the volatiles produced by both species were remarkably similar, the presence of (*E*)-2-decenal in *T. c. accerra* but not *T. pallidovirens* extracts suggests that this compound may function as a component of a species-specific pheromone signal.

The low numbers of bugs caught in field trials has hampered clarification of many details of the pheromone blend. The low trap catches may reflect an inherently weaker attraction to pheromones than is typically seen with other groups of insects such as Lepidoptera, for several reasons. First, low trap catches have been observed with several phytophagous stink bug species, including *Nezara* spp. (Aldrich et al., 1993), *Euschistus* spp. (Aldrich et al., 1991, 1995; Borges et al., 1998;), *Piezodorus* spp. (Borges et al., 1998), *Biprorulus bibax* (James et al., 1996), and *Acrosternum hilare* (McBrien and Millar, unpublished data). Second, a specialist predator of *T. pallidovirens*, the wasp *Astata occidentalis* Cresson (Hymenoptera: Sphecidae) was attracted to synthetic *T. pallidovirens* pheromone (Millar et al., 2001), indicating that the chemistry of the synthetic pheromone was correct and that the dose tested was within a biologically relevant range. Third, phytophagous stink bugs mate numerous times during their lifetimes of weeks to months, during which they feed continuously. Consequently, there is less

urgency for stink bugs to mate than for ephemeral insects with nonfeeding adult stages, which must find mates quickly before they exhaust their energy reserves and die.

The two-part mate location behavior, mediated by pheromonal and vibrational cues, also may have contributed to low trap captures. Pheromone alone, in the absence of the vibrational signals, may provide little inducement for bugs to orient towards and enter a trap. Consequently, our trap captures may represent only a fraction of the insects attracted to the vicinity of a pheromone-baited trap. Furthermore, *T. pallidovirens* and other phytophagous pentatomids [*A. hilare* (McBrien et al., 2001), and *Chlorochroa* spp. (Ho and Millar, 2001a,b)] responding to pheromone lures showed little inclination to fly. This suggests two possibilities. First, the pheromones may act over relatively short range, because a responder would be unlikely to walk more than a few meters. Second, the pheromone may attract a responder to the general vicinity of a pheromone producer, with subsequent mate location behaviors being mediated by substrate-borne vibrations. Thus, a responding insect using the pheromone to orient to the vicinity of a pheromone producer must be on a substrate rather than flying in order to detect the vibrational signals that mediate the final steps of mate location.

The fact that *T. pallidovirens* are attracted by $E2,Z4,Z6$–10:COOMe and any one of the three sesquiterpenes has practical implications for commercial formulation of lures. Furthermore, bugs were indifferent to the (−)-enantiomer in formulations containing racemic α-curcumene, and to *epi*-sesquiphellandrene in formulations containing synthesized (−)-β-sesquiphellandrene, so that these cheaper stereoisomeric mixtures can be used in lures. Even blends of $E2,Z4,Z6$–10:COOMe with the sesquiterpene fraction of commercial ginger oil, containing approximately 70% of the three bug-produced compounds, were attractive. The fact that these baits, and baits formulated with one to three sesquiterpenes, either pure or as mixtures of stereoisomers, attracted female *T. pallidovirens* illustrates the flexibility in the responses of these insects to a wide palette of pheromone formulations.

In summary, male *T. pallidovirens* produce a sex pheromone consisting of at least two and as many as four components. The pheromone attracted only females in all types of bioassays used, with no indication that males or immatures were attracted. Trap catches were low, and further development of traps and lures will be required to produce an effective system for sampling *T. pallidovirens* populations. Because stink bugs use both pheromonal and vibrational signals for mate location, it may be necessary to incorporate both types of signals into trap designs.

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EFFECTS OF RELATIVE HUMIDITY, TEMPERATURE, AND POPULATION DENSITY ON PRODUCTION OF CUTICULAR HYDROCARBONS IN HOUSEFLY *Musca domestica* L.

NICO NOORMAN* and CORNELIS J. DEN OTTER

Group Sense Organs and Behaviour
Department of Animal Physiology
University of Groningen
P.O. Box 14, 9750 AA Haren. The Netherlands

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Abstract—The production of cuticular hydrocarbons by both males and females of *Musca domestica* L. under very wet conditions (90% relative humidity) compared to the production at 50 and 20% relative humidity is delayed up to at least 3 days after emergence from the pupae. Eight days after emergence, however, males contain the same amounts of hydrocarbons at 90, 50, and 20% relative humidity, whereas females at 90% still possess less of these substances than at 50 and 20%. It is suggested that this is due to the fact that males, being more active than females, need more cuticular hydrocarbons to prevent water loss. No indication is found that relative humidity has a different effect on the production of the sex pheromone, muscalure [(*Z*)-9-tricosene] by females than on the production of the other hydrocarbons. Male and female flies produce more hydrocarbons at 35°C than at 20°C. On females, the relative amounts of nonacosane and methyl- and dimethylnonacosanes are significantly higher at 35°C than at 20°C. Female flies produce some (*Z*)-9-tricosene after eight generations at low population density in contrast to females at high population density, which did not produce muscalure. We suggest that because of the relatively large contribution to the total population, the properties of a small number of females are likely to be expressed sooner in the next generations of small populations than in those of large populations.

Key Words—*Musca domestica*, sex pheromone, muscalure, (*Z*)-9-tricosene, cuticular hydrocarbons, environmental effects.

*To whom correspondence should be addressed. E-mail: N.Noorman@biol.rug.nl

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INTRODUCTION

The cuticular hydrocarbons of insects provide a barrier to water diffusion, thus preventing desiccation of the animals. However, some of these substances may also act as semiochemicals and may play a role in mating behavior. In houseflies, several hydrocarbons on the body surface of females induce sexual behavior in males. (*Z*)-9-Tricosene is considered to be the main component of this sex pheromone (Carlson et al., 1971, 1974; Mansingh et al., 1972; Richter et al., 1976; Uebel et al., 1976; Rogoff et al., 1980; La-France et al., 1989; Adams et al., 1995).

It is known that environmental factors may affect the production of cuticular hydrocarbons (Hadley, 1977, 1978; Toolson et al., 1990; Gibbs et al., 1991). In several species of arthropods, higher temperatures and lower humidities result in a greater proportion of cuticular *n*-alkanes relative to branched alkanes and/or unsaturated hydrocarbons. These differences in hydrocarbon composition account for concomitant differences in cuticular permeability to water (Toolson and Hadley, 1979; Hadley and Schultz, 1987). Gibbs et al. (1995) suggested that production of sex pheromone by *Musca domestica* females might increase cuticular transpiration. Because the pheromone components are unsaturated or methyl-branched, this might lead to higher permeability to water.

In previous studies (Noorman and Den Otter, 2001), we found that (*Z*)-9-tricosene comprised up to 20–30% of the total hydrocarbons on females of a strain that had been cultured for many generations in the laboratory at constant temperature and relative humidity. On wild-type females, however, less than 0.5% of this component was present. It appeared that on females of subsequent generations of laboratory populations, the quantities of both (*Z*)-9-tricosene and tricosane—the production processes of which were shown to be closely linked—increased, whereas the amounts of the other cuticular hydrocarbons remained the same. This led us to assume that although temperature and humidity may affect the production of all cuticular hydrocarbons, temperature and humidity mainly determine the quantities of the nonpheromonal components. (*Z*)-9-Tricosene, however, may be produced primarily as a result of a selection process in high-density populations in isolated environments, as females with higher quantities of this substance on their body are more attractive to males. These females may be the first to mate and oviposit fertile eggs. Their progeny may, therefore, survive in higher numbers in the next generation than those of females producing lower amounts of (*Z*)-9-tricosene. It may be expected that this selection proceeds faster in high-density than in low-density populations.

This paper presents results of studies designed to test these hypotheses. We investigated the effects of relative humidity, temperature, and population density on the production of various hydrocarbons in males and females of *Musca domestica*.

METHODS AND MATERIALS

Insects. Larvae of *Musca domestica* were obtained from a cow-house with pigsty (Pesse strain) and poultry breeding (Van Diermen strain) in the Netherlands. To investigate the effects of relative humidity on cuticular hydrocarbon production, experiments were carried out with Pesse flies cultured in the laboratory for about 40 generations (Pesse-40) at 25°C, 50% relative humidity, and 12L:12D photoregime. Eight-day-old females of this strain contained about 3 μg muscalure/fly.

Immediately after emergence, 10 male and 10 female flies were placed together in a cage ($15 \times 8 \times 5$ cm). Six cages were used, two of which were kept at about 90% relative humidity, two at 50% relative humidity, and two at about 20% relative humidity. In order to obtain 90% relative humidity, the cages were placed on a rack in the rearing room in a Perspex container ($50 \times 50 \times 50$ cm), the bottom of which was covered with a layer of tap water. Ventilation took place through holes in the walls of the container. To achieve 20% relative humidity, the bottom of the container was covered with oven-dried silicagel, which was replaced before it was saturated with water. For 50% relative humidity, the cages were placed in an empty container from which the upper cover was removed. Every day the actual temperature and relative humidity in the cages were recorded. The temperature and relative humidity in the cages were 25.8 ± 0.5°C, and 19.1 ± 1.5%, 47.2 ± 1.4% and 91.8 ± 2.0%, respectively. Hydrocarbon quantities were determined on flies of each of these groups 3 and 8 days after emergence.

To establish the effects of temperature, Pesse flies were used from a strain reared for about 30 generations (Pesse-30) in the laboratory at 25°C, 50% relative humidity, and 12L : 12D photoregime. Eight-day-old females of this strain contained about 1 μg muscalure/fly. Hydrocarbon quantities of 8-day-old flies from two cultures were compared. Immediately after emergence, the flies of these cultures had been placed at 20 and 35°C, respectively, in cages ($30 \times 30 \times 40$ cm) containing about 100 males and females.

The effects of population density on cuticular hydrocarbon composition were studied using Van Diermen flies. Flies originating from the first generation grown in the laboratory were reared for 8 successive generations (F_1–F_8) at 25°C, 50% relative humidity, and 12L : 12D photoregime in cages ($30 \times 30 \times 40$ cm) containing either less than 20 or more than 300 flies. Hydrocarbon quantities on 8-day-old flies of the F_1 and F_8 generations were compared.

All flies were fed a mixture of powdered milk, sugar, and yeast (5:5:1 by weight). Water was present ad libitum in a tube filled with cotton. In the first two series of experiments, flies of only one generation that already produced considerable amounts of muscalure were studied, because we expected an immediate effect on cuticular hydrocarbon production when changing the abiotic factors of

temperature and relative humidity. Since, however, the amount of (*Z*)-9-tricosene production is supposed to be primarily the result of a selection process in isolated high-density populations, we compared, in the third series, flies from two generations, starting with a culture that did not produce detectable amounts of (*Z*)-9-tricosene.

Chemical Analysis. Individual flies were immersed in 0.2 ml hexane and shaken for 1 min, to bring the hexane in full contact with the whole cuticle, after which the fly was kept in this fluid for 1 hr for extraction of the cuticular hydrocarbons. The hydrocarbons were not separated from the nonhydrocarbon compounds because in *Musca domestica* the cuticular wax layer (above C_{20}) consists almost exclusively of hydrocarbons except for some oxygenated compounds (Mpuru et al., 2001).

Gas chromatography was performed on a Hewlett Packard 5890 series II gas chromatograph. Two microliter aliquots of individual extractions were injected into a WCOT fused silica CP-Sil-5 CB column (25 m $\times$ 0.32 mm ID; film thickness, 0.25 μm; Chrompack) with injector at 250°C and FID at 300°C. The flow rate of the helium carrier gas was 26 cm/sec. The split ratio was 56:1. GC oven temperature was programmed from 50 to 300°C at 10°C/min. 2-Nonanone was used as an internal standard. A calibration curve of this chemical in relation to some relevant hydrocarbons was made in order to calculate the proper amounts of the cuticular hydrocarbons. Muscalure and the other hydrocarbons were identified by comparing the retention time with reference runs of alkanes and (*Z*)-9-alkenes or with data from the literature. In an earlier study (Noorman and Den Otter, 2001), hydrocarbons were identified by mass spectrometry, and this indicated that the relevant hydrocarbons can be distinghuised by differences in retention times. Quantities of the hydrocarbons were expressed as percentages of the internal standard or as micrograms.

RESULTS

The total amounts of cuticular hydrocarbons on males and females, 3 and 8 days old, that had been kept at different relative humidities from emergence are presented in Figure 1. The amounts of hydrocarbons on both 3-day-old males and females were higher at 20 and 50% than on those kept at 90% relative humidity. (Mann-Whitney U test, $P < 0.01$). Eight days after emergence, the total hydrocarbon quantities on the flies became higher at all relative humidity levels compared to day 3 (Mann-Whitney U test, $P < 0.01$). In addition, the amounts on 8-day-old males at 90% relative humidity were no longer lower than on those kept at 50 and 20%. This is in contrast to the females of which 8-day-old specimens at 90% relative humidity still contained less hydrocarbons than those kept at the lower relative humidities. (Mann-Whitney U test, $P < 0.05$).

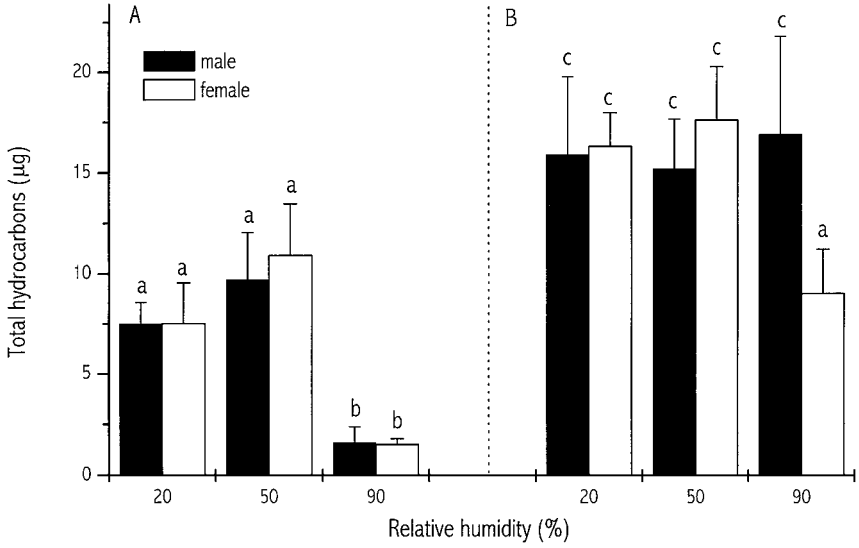


FIG. 1. Amounts of total cuticular hydrocarbons of males ($N = 5$) and females ($N = 10$) of *M. domestica*, 3 (A) and 8 (B) days after emergence, at different relative humidities. Error bars represent standard deviations. Averages labeled with different letters differ significantly.

The amount of the most abundant hydrocarbon compounds present on the cuticle of the flies is about 90% of the total cuticular hydrocarbons (Figure 2). The hydrocarbon profiles of males and females differed considerably; males had relatively high quantities of (*Z*)-9-heptacosene, whereas females had relatively high amounts of (*Z*)-9-tricosene, tricosane, and (Z)-9-heptacosene. On day 8, the amounts of hydrocarbons were higher than on day 3, but the hydrocarbon profiles of the males and females were still about the same. The amounts of (*Z*)-9-tricosene plus tricosane on females are about 15% of the total cuticular hydrocarbon quantity on day 3 after emergence and about 30% of the total on day 8 at each of the three relative humidities.

Striking differences in the total amounts of hydrocarbons on 8-day-old male and female flies were present between flies kept at 20°C and at 35°C (Figure 3). Males at 35°C produced about 80% more hydrocarbons than males kept at 20°C (19.5 and 10.7 μg, respectively); females at 35°C produced about 50% more than females kept at 20°C (21.2 and 13.9 μg, respectively). In male flies, the relative amounts of the various hydrocarbons produced at the two temperatures did not differ significantly, whereas in female flies the relative amounts of nonacosane and of the methyl- and dimethylnonacosanes were higher at 35°C than at 20°C

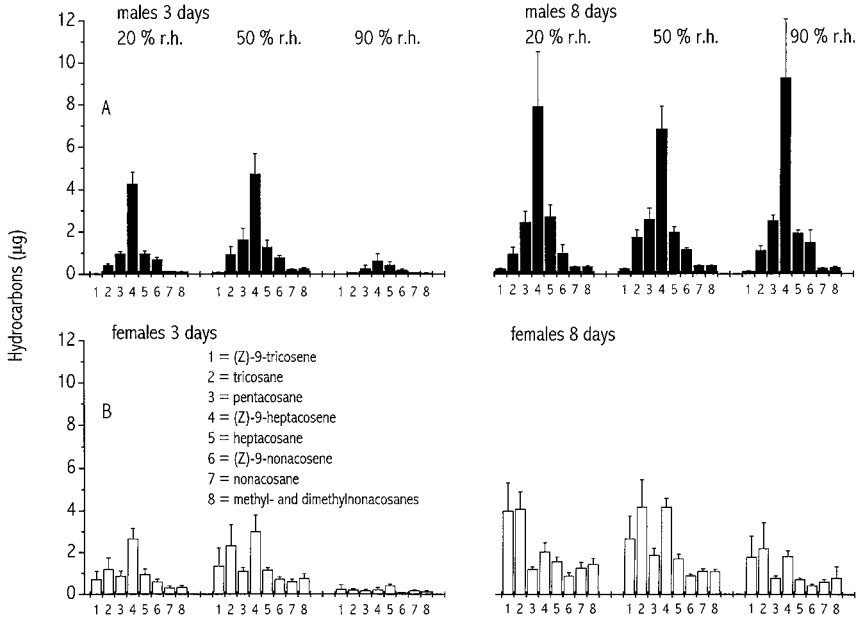


FIG. 2. Amounts of cuticular hydrocarbons on males (A, $N = 5$) and females (B, $N = 10$) of *M. domestica*, 3 and 8 days after emergence, at different relative humidities. Error bars denote standard deviations.

(t test, $P < 0.01$). Although at 20°C females appeared to produce less muscalure than at 35°C (1.0 vs. 1.4 μg), this difference was not significant. The relative amounts of the remaining hydrocarbons of females kept at 20°C and at 35°C did not differ. Hence, the production of muscalure is not different from the production of these hydrocarbons at the two temperatures.

The total amounts of the hydrocarbons on the first generation wild-type Van Diermen males (11.9 μg) and females (16.5 μg) did not differ from those on the eighth generation flies kept at low or high density (males 11.4 and 14.3 μg, females 19.2 and 14.0 μg, respectively, Mann-Whitney U test, $P > 0.05$) (Figure 4). The hydrocarbon profiles of the males were similar to those of the males of the Pesse strains used in the previous experiments (cf. Figures 2A, 3A, and 4A), whereas those of the females differed considerably from the Pesse females (cf. Figures 2B, 3B, and 4B). On 8-day-old Van Diermen females, nonacosane and methyl- and dimethylnonacosanes were the most abundant hydrocarbons. Females of the low-density population contained (*Z*)-9-tricosene, although the amounts were low.

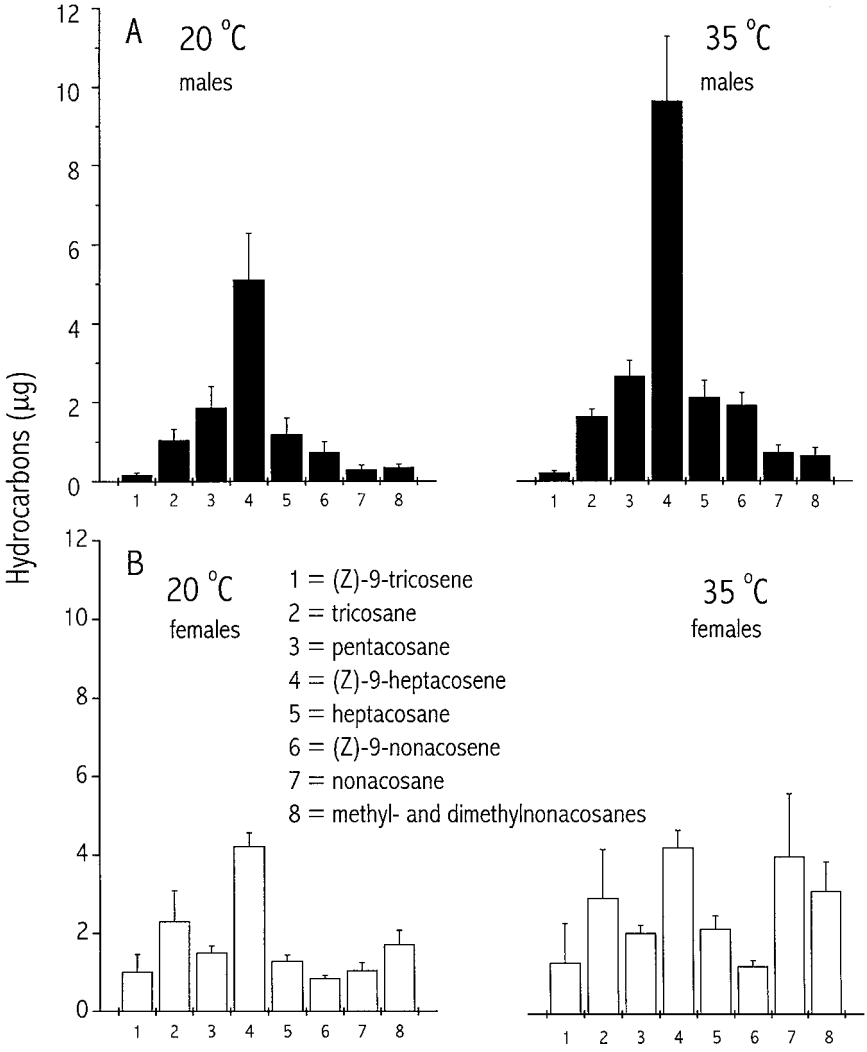


FIG. 3. Amounts of cuticular hydrocarbons on 8-day-old males (A, $N = 5$) and females (B, $N = 10$) of *M. domestica* at 20 and 35°C. Error bars denote standard deviations.

DISCUSSION

Evaporation of water through the cuticle can be expected to be lower at higher relative humidity. Therefore, smaller amounts of cuticular hydrocarbons may be needed to protect the flies from desiccation. Toolson and Hadley (1979)

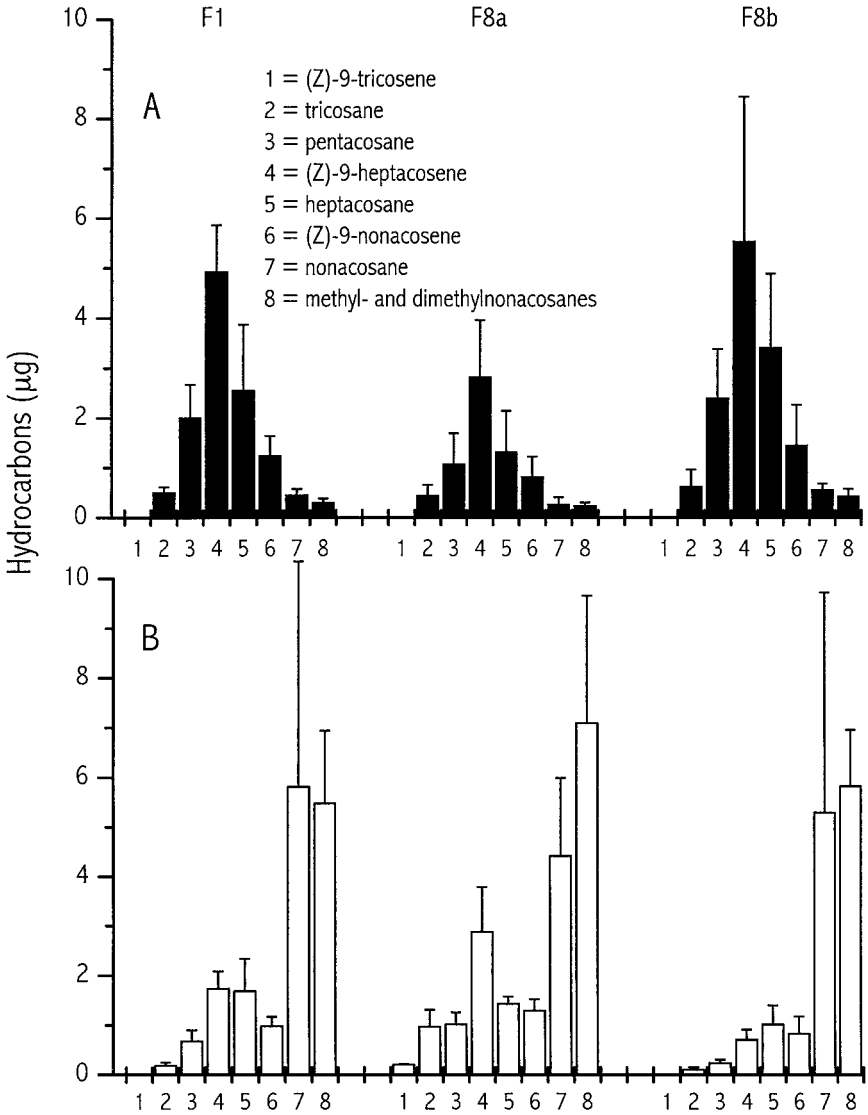


FIG. 4. Amounts of cuticular hydrocarbons on 8-day-old first laboratory generation (F1) males (A) and females (B) of *M. domestica* and on males and females of the same strain kept in culture for eight generations at different population densities (F8a <20 and F8b 300 flies/cage). $N = 10$ for each generation. Error bars indicate standard deviations.

observed marked seasonal changes in the relative abundance of cuticular hydrocarbons of the scorpion *Centruroides sculpturatus*, the percentage of *n*-alkanes in the epicuticular hydrocarbon decreasing considerably in warmer months, whereas the proportion of branched alkanes increased. Hadley and Schultz (1987) showed a correlation between water loss rates and quantities and composition of cuticular hydrocarbons. In the tiger beetle, *Cicindela obsoleta*, a summer-active species that inhabits dry grasslands, the cuticular hydrocarbon fraction only contains saturated hydrocarbons, whereas on the body of *C. oregona*, a spring- and fall-active species that lives along water courses, about 50% of the hydrocarbons are unsaturated. The presence of unsaturated hydrocarbons is thought to increase the permeability of the hydrocarbon layer to water. Indeed, Hadley and Schultz (1987) found a significant negative correlation between water loss rate and the quantity of saturated hydrocarbons.

We found that both males and females of 3 days old flies reared at 90% relative humidity possessed remarkably lower amounts of saturated and unsaturated hydrocarbons than those reared at 20 and 50%. Eight days after emergence, however, males kept at 90% relative humidity contained the same amounts of hydrocarbons as those kept at 20 and 50%, whereas females still had less hydrocarbons on their cuticle. A possible explanation may be that, in general, males are more active than females, mainly due to intensive mating behavior (personal observations). As a consequence, males may need more cuticular hydrocarbons to prevent water loss than less active females. The hydrocarbon profiles of the females cultured at different relative humidities are about the same. This suggests that the production of (*Z*)-9-tricosene, the main component of the female sex pheromone, may not be affected by changes in humidity to the same extent as of the other hydrocarbons.

The melting temperatures of the alkenes, methylalkanes, and alkanes isolated from 4-day-old houseflies are -0.2, 29.9, and 41.5°C, respectively (Gibbs et al., 1995). The melting temperature (midpoint of phase transition) of the mixture of all cuticular hydrocarbons was 36.8°C in females and 39.4°C in males. However, the transition of lipids from the solid to the liquid phase ranges over 10–15°C. The necessity to keep the melting temperature of the cuticular lipid mixture within a certain range can probably affect the amounts in which certain hydrocarbons are produced, depending on different environmental circumstances. We observed that the relative amounts of nonacosane and methylnonacosanes on females were higher at 35°C than at 20°C. This may have led to an increase of the melting temperature of the whole mixture of cuticular hydrocarbons. The hydrocarbon profiles on male flies were, however, the same at the two temperatures. The melting temperature of all cuticular hydrocarbons is higher in males than in females (Gibbs et al., 1995). Probably there is no reason to change the amount and composition of hydrocarbons on males in an environmental temperature range of 20°C to 35°C.

In the low-density population cultures (<20 flies/cage), all females of the eighth generation produced a low amount of muscalure, whereas no muscalure was found on the high-density (>300 flies/cage) females. We were able to compare eight generations, but nevertheless, after this limited number of generations, differences in (*Z*)-9-tricosene production between the two populations could already be observed. We found (*Z*)-9-tricosene on all the females of the low-density population analyzed, and each fly contained about the same amount of this compound. Surprisingly, none of the females of the high-density populations produced detectable amounts of (*Z*)-9-tricosene. Probably, this difference developed by chance. The properties of one or a few females are likely to be expressed in relatively more individuals in the next generations of small (laboratory culture populations) than in large (wild-type) populations. It is shown that selection can sneak in rapidly. This effect should always be a point of attention in laboratory colonies.

The clear difference in the hydrocarbon profiles between 8-day-old females of the Pesse-30 and -40 strains and the Van Diermen strain may be explained by focusing on the main function of cuticular lipids in general, i.e., to provide a barrier to water loss. The insects will need a certain amount of cuticular lipids in order to regulate water balance in an effective way. When (*Z*)-9-tricosene and tricosane in female houseflies are present only in small amounts, this is compensated for by other hydrocarbons (nonacosane and methyl- and dimethylnonacosanes). This assumption seems plausible when considering the total amount of hydrocarbons on females of the Pesse-40 and Van Diermen strains in the experiments carried out at the same temperature and humidity (25°C, 50% relative humidity). Females of both populations produced about the same amount of total hydrocarbons irrespective of the hydrocarbon profiles. In previous studies, Noorman and Den Otter (2001) also found that both first-generation Van Diermen and Pesse females in the laboratory produced low or undetectable amounts of (*Z*)-9-tricosene and relatively high amounts of nonacosane and methyl- and dimethylnonacosanes. Since no significant differences in the hydrocarbon profiles between these first-generation females of the Pesse and Van Diermen strains existed, strain differences do not appear to be responsible for the differences in hydrocarbon profiles.

The overall conclusions of the experiments are that relative humidity and temperature play a prominent role in the production of cuticular hydrocarbons and that the effects on the production of muscalure and the other hydrocarbons are similar. The population-density experiment showed that in a small number of generations selection might give rise to differences in muscalure production among populations. The fact that, in contrast to high-density population females, the low-density population cultures produced some muscalure suggests that population density is not the main factor for inducing muscalure production.

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GERANIAL: THE ALARM PHEROMONE IN THE NYMPHAL STAGE OF THE ORIBATID MITE, *Nothrus palustris*[1]

SATOSHI SHIMANO,[2,*] TOMOYO SAKATA,[3] YOSHIKATSU MIZUTANI,[2] YASUMASA KUWAHARA,[3] and JUN-ICHI AOKI[2]

[2]*Department of Soil Zoology*
Institute of Environmental Science and Technology
Yokohama National University
Hodogaya-ku, Yokohama 240-8501, Japan

[3]*Laboratory of Chemical Ecology*
Division of Applied Life Sciences
Graduate School of Agriculture, Kyoto University
Sakyo-ku, Kyoto 600-8502, Japan

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Abstract—The presence of an alarm pheromone was demonstrated in the deutonymphal stage of the oribatid mite, *Nothrus palustris* (C. L. Koch). The active principle was identified as 3,7-dimethyl-(*E*)-2,6-octadienal, geranial, and was shown to be active at doses of 10–100 ng. This is the first such pheromone identified in oribatid mites.

Key Words—Alarm pheromone, geranial, oribatid mites, *Nothrus palustris*, opisthonotal gland.

INTRODUCTION

While maintaining a culture of *Nothrus palustris* (C. L. Koch) (Oribatida: Nothridae), which was started from field-collected deutonymphs, the following phenomena were noticed: (1) the species tended to aggregate into a colony when feeding on fallen leaves or filter paper, (2) if one or several mites at the center of the aggregation were crushed with forceps or a needle, aggregating mites around them started to escape, and the aggregation became loose within several minutes. The same escape behavior was reproducible when a small filter paper (3×3 mm)

* To whom correspondence should be addressed at Department of Upland Farming, National Agricultural Research Center for Tohoku Region, Arai, Fukushima, Fukushima 960-2156, Japan. E-mail: satoshis@affrc.go.jp

[1]Chemical Ecology of Oribatid Mites II. For the previous paper, see Sakata et al. (1995).

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impregnated with the hexane extracts of nymphal bodies was placed at the center of the aggregation. These finding suggested that deutonymphs of the species possess an alarm pheromone that is discharged as a volatile compound on being crushed, possibly from a pair of opisthonotal glands (Howard et al., 1988), and that the active compound is extractable from nymphal bodies by hexane.

We report here identification of the alarm pheromone of the oribatid mite as 3,7-dimethyl-(*E*)-2,6-octadienal, geranial. The compound seems to be widely distributed among Oribatida (Sakata et al., unpublished data), whereas its geometric isomer 3,7-dimethyl-(*Z*)-2,6-octadienal, neral, is a major compound distributed widely among Astigmata (Kuwahara, 1999).

METHODS AND MATERIALS

Mites. About 200 individuals of *Nothrus palustris* (C.L. Koch) (Oribatida: Nothridae), consisting mainly of deutonymphs together with larvae, protonymphs, and tritonymphs, were collected on August 10, 1996, from litter and soil on the campus of Yokohama National University, Hodogaya-ku, Yokohama, Japan, by Tullgren funnel extraction. The species inhabits forest litter as a decomposer and has been recorded in many parts of the Palaeartic region (e.g., Sellnick and Forsslund, 1955). Nymphs were raised to adults in the laboratory by feeding with forest litter. Adults were used to confirm the species, because it was difficult to identify the species in the nymphal stages. Mites at these stages were white in color and had a fragile appearance, unlike the well sclerotized adults.

Bioassay. Groups of 20 deutonymphs were transferred into containers comprised of open glass tubes (1 cm diam. $\times$ 2 cm high) with a glass plate as the bottom. Glass Petri dishes (9 cm diam.) were inverted and used as plates. The container was set up on a circle (1 cm diam.) drawn underneath the Petri dish. Mites in the container were allowed to settle for (10–20 minutes) to offset handling effects, then the glass tubes were removed with minimal disturbance. At the center of the circle in which mites were left motionless, either a filter paper (3×3 mm) with a candidate compound dissolved in 3 μl of hexane or a freshly crushed mite body was introduced. Numbers of mites remaining within the circle were then counted every minute for 5 min. Filter paper impregnated with hexane (3 μl) alone was used as a control.

Instruments. Gas–liquid chromatography (GC) was carried out on a Hewlett Packard HP-5890 series II Plus equipped with a flame ionization detector. An HP-5 capillary column (0.32 mm diam. $\times$30 m, 0.33 μm film thickness) was used at a temperature program of 60°C to 290°C at 10°C/min, with an initial 2 min hold at 60°C. Samples were analyzed in splitless mode using He at 1.23 ml/min as the carrier gas. The chromatogram was processed with an HP 3396 series II integrator. GC-mass spectrometry (GC-MS) was performed on an Hewlett Packard 5989B

mass spectrometer coupled with an HP-5890 series II Plus gas chromatograph in splitless mode, using the same capillary column and conditions as described above.

Preparation of Hexane Extract. A mite (a deutonymph, protonymph, tritonymph, or an adult) was transferred with a needle into a small conical-bottomed tube (handmade, 8 mm diam. × 30 mm long) and 3 μl of hexane was added. The resulting hexane rinse was collected with a microsyringe at 3 min after introduction of the solvent. Then, all portions of the rinse were subjected to either GC or GC-MS analysis. Similar hexane rinses from a substantial number of mites (not counted) were used for preliminary bioassay and silica gel (Wakogel C-200) column chromatography.

RESULTS AND DISCUSSION

Upon exposure to a piece of filter paper impregnated with the hexane rinse (3 μl) prepared from a deutonymph, tested deutonymphs showed escape behavior from the arena, possibly due to the presence of the alarm pheromone. GC analyses of the mite extract revealed that each hexane rinse from all nymphal stages (protonymph, deutonymph, and tritonymph) indicated the same profile (Figure 1). The extract consisted of at least four components (compound A, t_R 2.82 min; compound B, t_R 6.59 min; compound C, t_R 7.12 min; and compound D, t_R 16.50 min), of which compound C was the major component. Compounds A, B, and D were minor components at nymphal stages. In contrast, the profile from the adult rinse was different from those of nymphs. Compound A was the major component, followed by compound D. Compound C was a trace component together with many unknown peaks (Figure 1). Although reliable GC profiles were not obtained from mites at larval stages, possibly due to their small body size, the same profile as that of nymphs was assumed. This is the first example of an oribatid mite in which the that major components in the extract (possibly the exudates from the oil gland) change through development from nymphal to adult stages.

Compound A (t_R, 2.82 min) showed an M^+ ion peak at m/z 152 (18%) and the base ion peak at m/z 109 (100%) with the following diagnostic ions at m/z 124 (44%), 94 (58%), 79 (77%), 77 (23%), and 43 (60%). This mass spectrum was identical to that of dehydrocineole (Ayorinde et al., 1984), and its t_R was also the same as that of the compound present in the astigmatid mite *Caloglyphus rodriguezi*. Consequently, compound A was identified as dehydrocineole. Among astigmatid mites, the compound is distributed only among species belonging to the genera *Caloglyphus*, *Schwiebea*, and *Rhizoglyphus* (Kuwahara, 1991, 1995, 1999).

Compounds B (t_R 6.59 min) and C (t_R 7.12 min) indicated both identical tRs to those derived from the standard citral, a mixture of 3,7-dimethyl-(*E*)-2,6-octadienal, geranial, and 3,7-dimethyl-(*Z*)-2,6-octadienal, neral. These compounds also showed the same characteristic GC-MS spectra with an M^+ ion each at

m/z 152 (3% and 9%) and the same base ion at m/z 69 (both, 100%) and were identical to those reported previously (Kuwahara et al., 1980). Compounds B and C were, thus, identified as neral and geranial, respectively.

Compound D showed an M^+ ion at m/z 292 (16%) and the base ion at m/z 55 (100%) with the following diagnostic ions at m/z 222 (2%), 208 (4%), 194 (5%), 180 (7%), 166 (10%), 152 (14%), 138 (16%), 124 (26%), 110 (35%), 96 (78%), 82 (80%), 69 (58%), and 41 (60%). Although the mass spectrum and t_R were identical to those obtained from a standard of 6,9-heneicosadiene from the astigmatid mite *Suidasia medanensis* as a minor component (Kuwahara, 1991, 1995, 1999), double bond positions and their geometry remain obscure.

When the hexane rinse was subjected to SiO_2 column chromatography and separated by elution with a sequence of ether–hexane mixtures, the alarm pheromone activity was recovered in the 10% ether/hexane eluate. The active fraction consisted of both compounds B and C, confirmed by GC and GC-MS analyses. The SiO_2 column chromatographic behaviors of these two compounds also coincided with that of citral. The isomeric purity of geranial was calculated as more than 90% among the nymphal extracts (Figure 1).

The alarm pheromone activity demonstrated by a squashed deutonymph coincided with that of the filter paper impregnated with doses of 10–100 ng of geranial (Table 1). The actual quantity of geranial determined by GC analysis was 91.4 ± 20.4 ng (mean $\pm$ SD, $N = 10$) per deutonymph, and this value was consistent with the bioassay results. Although there were no differences in the behavior of mites between exposure to 0.1 ng of synthetic genarial (isomer purity $>95\%$) and the control, escape behavior was observed to synthetic geranial at doses >1 ng. No activity was observed in response to synthetic neral (isomer purity $>95\%$), which was at least 10-fold less active than geranial.

In summary, the alarm pheromone found in deutonymphs of *Nothrus palustris* was identified as geranial. As the compound was also detectable in the extract from mites at all nymphal stages as the major component (Figure 1), its pheromonal

TABLE 1. ALARM PHEROMONE ACTIVITY OF A NYMPHAL BODY OF *Nothrus palustris* AND GERANIAL

Time after introduction of sample (min.)	Mites staying in test circle (N)					
		Geranial (ng)				Squashed mite body
	Control	0.1	1	10	100	
0	20	20	20	20	20	20
1	17	17	12	7	5	11
2	15	14	8	4	2	6
3	13	13	5	2	0	4
4	12	13	3	2	0	0
5	10	11	2	0	0	0

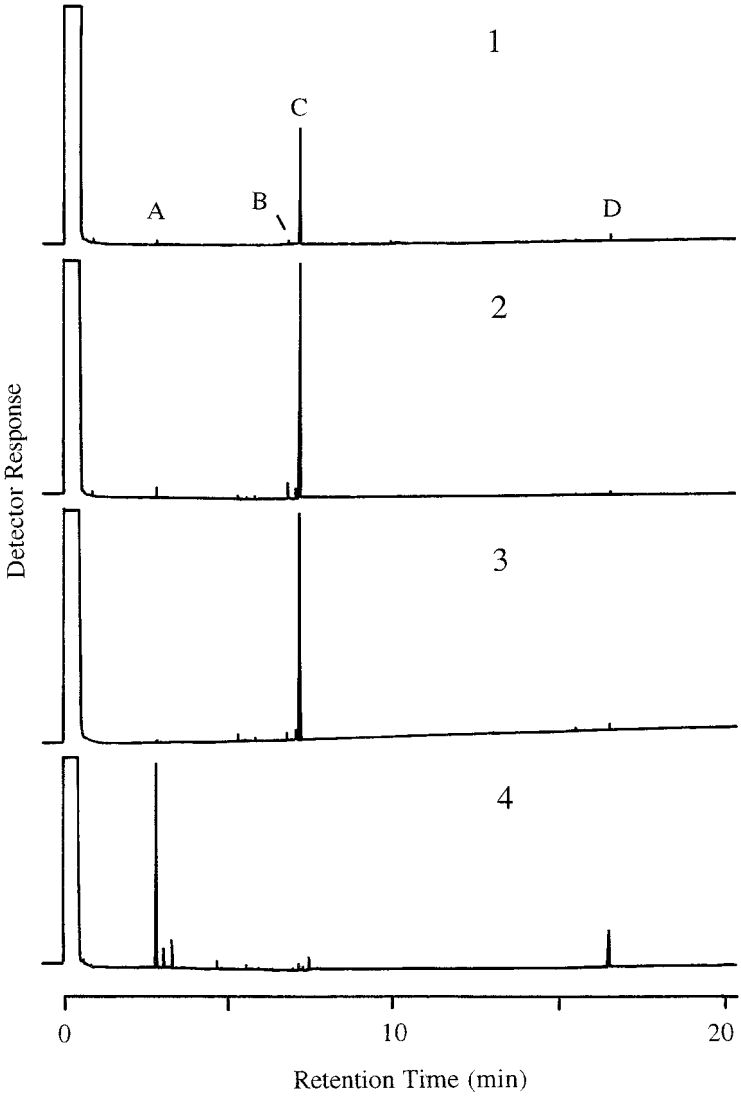


FIG. 1. Typical gas–liquid chromatograms of hexane extract of *Nothrus palustris* on an HP-5 fused silica capillary column at a temperature programmed condition from 60°C to 290°C at 10°C/min. 1: from a protonymph, 2: from a deutonymph, 3: from a tritonymph and 4: from an adult. Peak A: dehydrocineole, peak B: neral, peak C: geranial, and peak D: heneicosadiene.

function was also presumed to be present in mites at all stages. Although adults exhibited the same escape behavior on exposure to geranial at 100 ng, the GC profile was completely different to that of nymphal stages. Geranial was only a trace component in the extract, and dehydrocineole was the major component, followed by 6,9-heneicosadiene as the second largest. It is, therefore, unlikely that geranial functions as the alarm pheromone in adults. The reason for the adult behavior against high doses of geranial has yet to be clarified.

Mites at the nymphal stages were white in color and their epidermis was soft and fragile, similar to those of Astigmata. This might be why the alarm pheromone is required for nymphal stages, i.e, to minimize physical damage caused by natural enemies, similar to the case in Astigmata. On the other hand, adults possess a fully sclerotic brown epidermis that may protect the mites from various ecological pressures and may lessen their dependence on the alarm pheromone.

This is the first report of a semiochemical identified in an oribatid mite, possibly as a component of a pair of oil glands (the opisthonotal glands). The same gland is distributed among four cohorts of Oribatida: Parhyposomata, Mixonomata, Desmonomata, and Brachypylina (Norton, 1994). These glands have been shown to produce monoterpenes (Sakata et al., 1995, Raspotnig et al., 2002) and aromatics (3-ethyl phenol, γ-acaridial, and 1-methyl-2-naphtol) (Sakata and Norton, 2001), together with various hydrocarbons. However, their biological functions have not previously been clarified. Many studies of the ecology and biology of this species as a model of oribatid mites were reviewed by Krivolutsky et al. (1995) and Lebrun (1970), but the occurrence of an alarm pheromone has not been reported previously. The present identification of the alarm pheromone is the first step in the semiochemical study on the oil glands of Oribatida. Many other examples will likely be found in future studies. In the case of astigmatid mites, three kinds of pheromones are known in the total of 52 species examined: the alarm pheromone in 16 species, the aggregation pheromone in 2 species, and the sex pheromone in 5 species (Kuwahara, 1999). This is also the first species in which intrinsic differences were observed in the major component between adult and nymphal stages.

Among the 52 species of astigmatid mites examined to date, neral-rich citral has been shown to be distributed in 28 species, and neral functions as the alarm pheromone in five species. Geranial-rich citral is found in six species [(two species of Histiostomatidae, and in *Dermatophagoides pteronyssinus*, *Chaetodactylus nipponicus*, *Tyrophagus neiswanderi*, and *Schwiebea* sp. (Chiba)], among which geranial functions as the active principle of the alarm pheromone in *Histiostoma laboratorium*. Neral is the major component of citral among Astigmata (Kuwahara, 1999). On the other hand, among Oribatida geranial seemes to be the more dominant component, although current information regarding the neral–geranial ratio in Oribatida is limited (Sakata et al., unpublished data). This is the second example of geranial functioning as the alarm pheromone in two suborders, Astigmata and Oribatida, and the first example of the pheromone present in Oribatida.

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ENANTIOSPECIFIC ANTENNAL RESPONSE OF BARK BEETLES TO SPIROACETAL (*E*)-CONOPHTHORIN

QING-HE ZHANG,[1,*] TILL TOLASCH,[2] FREDRIK SCHLYTER,[1] and WITTKO FRANCKE[2]

[1]*Chemical Ecology, Department of Crop Science*
Swedish University of Agricultural Sciences
P.O. Box 44, SE-230 53 Alnarp, Sweden
[2]*Universität Hamburg, Institut für Organische Chemie*
Martin-Luther-King-Platz 6, D-20146 Hamburg, Germany

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Abstract—Optically pure synthetic enantiomers of (*E*)-conophthorin [(*E*)-7-methyl-1,6-dioxaspiro[4.5]decane], one of the volatiles affecting coniferophagous bark beetles, were tested on antennae of *Ips typographus, I. duplicatus, I. subelongatus, Dendroctonus micans*, and five *Scolytus* spp. by using combined gas chromatography–electroantennographic detection (GC-EAD). EAD dose–responses indicated that all three *Ips* species perceived only the naturally occurring and quantitatively dominant (5*S*,7*S*)-enantiomer, while its antipode, (5*R*,7*R*)-(*E*)-conophthorin was antennally inactive. Response thresholds for the *Ips* species were estimated as between 0.1 ng and 1 ng, or lower. The antennal responses of the *Ips* species caused by 100 ng of the (5*R*,7*R*)-enantiomer might be due to 1% impurity-(the active (5*S*,7*S*)-enantiomer) in the (5*R*,7*R*)-sample. At the 50-ng level, *D. micans* and five angiosperm *Scolytus* species (*S. intracatus, S. mali, S. ratzeburgi, S. rugulosus, and S. scolytus*) responded strongly to the (5*S*,7*S*)-enantiomer, while the (5*R*,7*R*)-enantiomer was antennally inactive. Currently updated knowledge on the natural occurrence, and electrophysiological and behavioral activity of (*E*)-conophthorin is summarized.

Key Words—*Ips typographus, Ips duplicatus, Ips subelongatus, Dendroctonus micans, Scolytus, Hylesinus, Phloeophthorus,* (*E*)-conophthorin, enantiomers, 1-hexanol, nonhost volatiles, antennae, GC-EAD, dose–response.

* To whom correspondence should be addressed at USDA-ARS Chemicals Affecting Insect Behavior. Laboratory, BARC-West, B-007, 10300 Baltimore Avenue, Beltsville, Maryland 20705, USA. E-mail: zhangq@ba.ars.usda.gov

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INTRODUCTION

Recent studies indicated that coniferophagous bark beetles not only recognize the aggregation pheromones of conspecifics as well as host kairomones, but are also able to perceive and avoid nonhost volatiles (NHVs) from angiosperm trees in their natural habitats (Huber, 2001; Zhang, 2001; Zhang and Schlyter, 2002, and in preparation). Among these, (*E*)-conophthorin, (*E*)-7-methyl-1,6-dioxaspiro[4.5]decane, is one of the major NHVs found in the bark of several angiosperm species in Europe [*Betula pendula* Roth., *B. pubescens* Ehrh. (Betulaceae), *Populus tremula* L. (Salicaceae), and *Quercus suber* L. (Fagaceae)], and in North America [*Populus tremuloides* Michx., *P. trichocarpa* Torr & A. Gray, *Acer macrophyllum* Pursh. (Aceraceae), and *Betula papyrifera* Marsh.] (Francke et al., 1995, Byers et al., 1998; Huber, et al., 1999, 2000a,b; Zhang et al., 2000). It elicited antennal responses from >10 species of coniferophagous bark beetle species (Zhang and Schlyter, in preparation) and caused a significant reduction in the response of more than 15 species of conifer-infesting scolytids to attractant-baited traps (Huber, 2001; Zhang, 2001; Zhang and Schlyter, in preparation). Quite recently, the combination of (*E*)-conophthorin with other NHVs and verbenone has shown potential for successful tree protection against mass attacks of *Dendroctonus ponderosae* Hopkins and *Ips typographus* (L.) (Huber and Borden, 2001; Jakuš et al., 2002).

(*E*)-Conophthorin was first identified as a male-specific compound in the ash bark beetle *Leperisinus fraxini* (F.) as well as in workers of the social wasps, *Paravespula vulgaris* (L.), *P. germanica* (F.), and *Dolichovespula saxonica* (F.) (Francke et al., 1978, 1979). It was later found to be a male-produced repellent pheromone of several species of Scolytidae (Coleoptera) (Kohnle et al., 1992; Pierce et al., 1995; Birgersson et al., 1995; Dallara et al., 2000; De Groot and DeBarr, 2000) and an aggregation pheromone component in two twig scolytids, *Pityophthorus* spp. (Dallara et al., 2000). A detailed compilation on the occurrence of spiroacetals in insects has been published recently (Francke and Kitching, 2001). Both the *E*- and *Z*-stereoisomer of conophthorin (Figure 1) were detected in various natural sources (both plants and insects), but the *E*-isomer was dominant in all taxa studied (with the *E*/*Z* ratios ranging from 2:1 to 40:1) (see Table 1 for details). The determination of the enantiomeric composition of (*E*)-conophthorin from natural sources indicated a strongly biased 5*S*,7*S*-configuration, with an *ee* of 30–100%.

The objective of the present investigation was to test whether the antennae of some angiosperm bark beetles (*Scolytus* spp.) and the spruce bark beetles, *Ips typographus* (L.), *I. duplicatus* (Sahlb.), and *Dendroctonus micans* (Kugelann), as well as the Asian larch bark beetle, *Ips subelongatus* Motschulsky [formerly recognized as *I. cembrae* (Heer) in the West; see Stauffer et al. (2001) for species status] would respond to both enantiomers of (*E*)-conophthorin or only detect

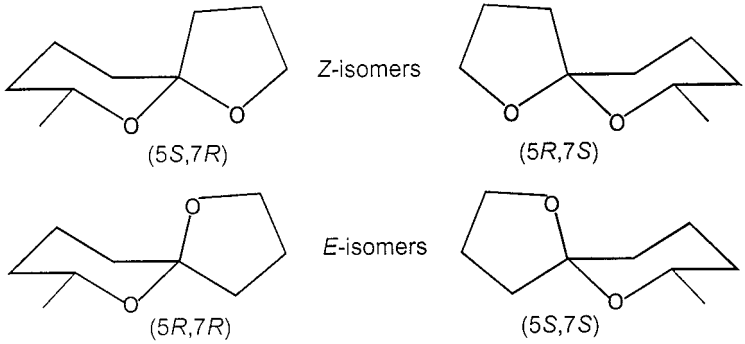


FIG. 1. Stereoisomers of conophthorin.

the major (5*S*,7*S*)-enantiomer. To answer this question, we used a combined gas chromatography–electroantennographic detection (GC-EAD) technique in a dose–response fashion, with optically pure synthetic enantiomers as well as GC-EAD and on-line enantiomeric separation of racemic (*E*)-conophthorin with a chiral stationary phase.

METHODS AND MATERIALS

Insects and Chemicals. Adults of *Ips typographus* and *I. duplicatus* were taken from laboratory cultures (It-114 and Id-05), originally collected from Norway and Inner Mongolia, China, respectively.*I. subelongatus* adults were collected directly from naturally attacked *Larix principis-rupprechtii* Mayr. trees, Inner Mongolia, China.

Dendroctonus micans (Kugelann) were collected from the bark of naturally infested Norway spruce, *Picea abies* Karst. at Clermont-Ferrand, France. Angiosperm bark beetles were obtained from naturally infested logs collected in the vicinity of Hamburg, Germany, which had been brought to the lab: *Hylesinus crenatus* (F.) in common ash, *Fraxinus excelsior* L.; *Phloeophthorus rhododactylus* (Marsh.) in broom, *Sarothamnus scoparius* (L.); *Scolytus intricatus* (Ratzeburg) in oak, *Quercus robur* L.; *S. mali* (Bechstein) in plum, *Prunus domestica* L.; *S. ratzeburgi* Jason in birch, *Betula pendula* Roth; *S. rugulosus* (Ratzeburg) in plum, *Prunus domestica* L.; and *S. scolytus* (F.) in elm, *Ulmus minor* Mill. Analyses for unambiguous identification of (*E*)-conophthorin in beetles was carried out by coupled gas chromatography–mass spectrometry (GC-MS) as described elsewhere (Francke et al., 1996).

Both enantiomers, (5*S*,7*S*)-(*E*)-conophthorin (97%; *ee* = 98%) and (5*R*,7*R*)-(*E*)-conophthorin (91%; *ee* = 98%), were synthesized according to the procedure

TABLE 1. NATURAL SOURCES AND ELECTROPHYSIOLOGICAL AND BEHAVIORAL ACTIVITY OF CONOPHTHORIN[a]

Insects and plants	Production						Activity: Electrophysiological			Activity: Behavioral			Ref.[b]
	E	*Z*	*E*/*Z*	5*S*,7*S*	5*R*,7*R*	5*S*,7*S ee* (%)	*E*	5*S*,7*S*	5*R*,7*R*	*E*	5*S*,7*S*	5*R*,7*R*	
Wasps (Hymenoptra: Vespidae)													
Paravespula vulgaris L.	√	√	7-10:1	√	√	*90-95*					▲		1, 2
P. germanica (F.)	√	√	35-40:1	√	√	*95*					▲		2
Dolichovespula saxonica (F.)	√	√	2:1	√	√	*30*					▲		2
Fruit fly (Diptera: Tephritidae)													
Bactrocera xanthodes (Broun)	√	√	20:1	√	√	*95*							3
Angiosperm scolytids (Col.: Scolytidae)													
***Hylesinus crenatus* F.**	√												4
Leperisinus fraxini Panzer [=*L. varius* (F.)]	√									▲	▲	▲	2, 5
***Phloeophthorus rhododactylus* (Marsh.)**	√												4
***Scolytus intricatus* Ratzeburg**							●	⊗					4
***S. mali* (Bechstein)**							●	⊗					4
***S. ratzeburgi* Janson**	√						●	⊗					4
***S. rugulosus* (Ratzeburg)**							●	⊗					4
***S. scolytus* (F.)**							●	⊗					4
Conifer scolytids (Col.: Scolytidae)													
Conophthorus conicolens Wood										▲			6
C. coniperda (Schwarz)	√	√	15:1	√	√	*95*				▲	▲		7, 8
C. ponderosae Hopkins	√			√						▲			9
C. resinosae Hopkins [=*C. banksianae* (McPherson)]	√	√	9-27:1	√	√	*96*				▲	▲	▲	10
C. teocotum Wood										▲			6
Cryphalus piceae (Ratz.)	√			√		*100*				▲	▲	⊗	5

***Dendroctonus micans* (Kugelann)**			●	●	⊗			4
D. ponderosae Hopkins	√		●			▲		11, 12
D. pseudotsugae Hopkins			●			▲		12, 13, 14
D. rufipennis (Kirby)			●			⊗		12, 13
Dryocoetes confusus Swaine			●			○▲		12, 13, 14
Ips cembrae (Heer)						▲		15
***I. duplicatus* (Sahlb.)**			●	●	⊗	▲		4, 16
I. pini (Say)			●			▲		12, 13, 14
I. sexdentatus Börner			●			▲		17
***I. subelongatus* Motschulsky**			●	●	⊗			4
***I. typographus* (L.)**			●	●	⊗	▲		4, 18, 19
***Orthotomicus erosus* (Woll.)**			●	●	⊗	▲		4, 17
Pityogenes bidentatus Herbst			●			⊗		20, 21
P. chalcographus (L.)						⊗		20
Pityophthorus cariniceps LeConte						○▲		22
P. carmeli Swaine	√	√					○	24
P. nitidulus (Mannerheim)	√	√					?	24
P. puberulus (LeConte)						⊗		22
P. setosus Blackman							▲	24
Taphrorychus bicolor Herbst	√							24
Tomicus destruens (Woll.)			⊗					21
T. minor (Hart.)			⊗			⊗		24
T. piniperda (L.)			⊗			⊗		24
Trypodendron domesticus L.			⊗					21
T. lineatum (Oliv.)			♀●♂⊗			⊗▲		5, 21

TABLE 1. CONTINUED

Insects and plants	Production						Activity: Electrophysiological			Activity: Behavioral			Ref.[b]
	E	*Z*	*E*/*Z*	5*S*,7*S*	5*R*,7*R*	5*S*,7*S ee* (%)	*E*	5*S*,7*S*	5*R*,7*R*	*E*	5*S*,7*S*	5*R*,7*R*	
Scolytid predators & associate insects													
Epuraea thoracica Tourn. (Col.: Nitidulidae)										○			5
Lascontus pertenuis Casey (Col.: Colydiidae)											○?		24
Thanasimus dubius (F.) (Col.; Cleridae)										⊗			22
T. formicarius (L.)							⊗			⊗			26
Other insects													
Hylobius abietis L. (Col.: Curculionidae)							●			▲			28
True bugs (Heteroptera)							●						14
Plants													
Acer macrophyllum Pursh (Aceraceae)	√			√		*?*							12
Betula pendula Roth (Betulaceae)	√			√		*?*							19, 27
B. pubescens Ehrh.	√	√	40:1	√	√	*80*							19
B. papyrifera Marsh.	√			√	√	*76*							12, 14
Dracaena fragrans (L.) Ker-Gawl. (Agavaceae)	√												29
Picea abies L. (Pinaceae)	√												23
Populus tremula (Salicaceae)	√			√		*?*							19
P. tremuloides Michx.	√			√	√	*80*							12, 14
P. trichocarpa Torr. and Gray	√			√	√	*84*							12, 14
Quercus suber L. (Fagaceae)	√			√									23

[a] Species studied in this paper are in bold type. √: production determined; ●: EAD active; ⊗: inactive; ▲: inhibitory; ○: attractive; ?: not known or unclear results;
[b] 1, Francke et al. (1978); 2, Francke et al., 1979; 3, Fletcher et al., 1992; 4, this paper ; 5, Kohnle et al., 1992; 6, Rappaport et al., 2000; 7, Bigersson et al., 1995; 8, De Groot et al., 1998; 9, Miller et al., 2000; 10, Pierce et al., 1995; 11, Francke et al., 1996; 12, Huber et al., 1999; 13, Huber et al., 2000a,b; 14, Huber, 2001; 15, Schlyter et al., 2001; 16, Zhang et al., 2001; 17, Jactel et al., unpublished; 18, Zhang, 2001; 19, Zhang et al., 2000; 20, Byers et al., 2000; 21, Zhang, 2001 (unpublished); 22, De Groot and DeBarr, 2000; 23, Francke et al., 1995; 24, Dallara et al., 2000; 25, Schlyter et al., 2000; 26, Zhang and Schlyter, unpublished; 27, Byers et al., 1998; 28, Schlyter et al., 2000 (unpublished); 29, Kaiser, 1993.

described by Jacobson et al. (1982). The stereogenic center at C-7 was introduced through the preparation of optically active 4-pentyne-2-ol (the chiral open chain component in Jacobson's synthesis) from acetylene and optically pure (2*S*)-methyloxirane (Aldrich). Racemic (*E*)-conophthorin (87%) was purchased from Phero-Tech. In addition, 1-hexanol (>98%; Aldrich), one of the active non-host green leaf volatiles (GLVs) in many *Ips* bark beetles (Zhang et al., 1999a,b), was used as an active control.

GC-EAD. GC-EAD analyses with *I. typographus*, *I. duplicatus*, and *I. subelongatus* were carried out in Sweden on antennae (with the severed head) of both sexes by using an HP 6890 gas chromatograph equipped with a 30-m × 0.25-mm × 0.25-μm fused silica column coated with HP-Innowax. A computerized electroantennodetection system (IDAC card and the program EAD ver. 2.3 from Syntech, Hilversum, The Netherlands), as described in detail by Zhang et al. (2000), served for antennal recordings. The tested (*E*)-conophthorin samples were dissolved in hexane at the following doses: 0.1, 1, 10, and 100 ng/μl. For the 1-hexanol active control, only the 100 ng/μl solution was used. For each GC-EAD run, ca. 2 μl of (*E*)-conophthorin solution plus ca. 2 μl of 100 ng/μl 1-hexanol (combined in the same syringe) were injected. Thus, after a 50:50 split in the GC, there should be ca. 100 ng of 1-hexanol and 0.1, 1, 10, and 100 ng of (*E*)-conophthorin (depending on the doses used) passing over the antennal preparations. The EAD responses were normalized to the relative response (%) to the active control.

GC-EAD analyses of *D. micans* and angiosperm bark beetles were carried out in Germany with cut off antennae of both sexes by using an HP 5890 GC with the same Syntech EAD system. Enantiomeric separation of (*E*)-conophthorin was achieved by using a 40-m × 0,25-mm-ID fused silica column coated with heptakis-*O*-(6-TBDMS-2,3-dimethyl)-β-cyclodextrin, operated at 100°C (*D. micans*) or 60°C for 3 min, then programmed to 160°C at a rate of 3°/min (*Scolytus* spp.).

RESULTS

Among angiosperm scolytids, (*E*)-conophthorin has earlier been reported from *Leperisinus fraxini* (Francke et al., 1979; Kohnle et al., 1992) and *Taphrorychus bicolor* (Dallara et al., 2000). We have now identified this spiroacetal in the frass produced by boring beetles of *Scolytus ratzeburgi, Phloeophthorus rhododactylus, and Hylesinus crenatus*. Since we could not find this compound in the respective host material, we suppose it to be beetle-produced.

In our EAD experiments, no antennal responses at the 0.1 ng level (i.e., 2 μl of 0.1 ng/μl solution injected) were found in *I. typographus* to either enantiomer or to the racemate of (*E*)-conophthorin [(±)-tC], while the antennal response to 100 ng of 1-hexanol (active control) was obvious (Figures 2 and 3A). When tested at the 1-ng level, the 5*S*,7*S*-enantiomer elicited significant antennal response to a level similar to that caused by 100 ng of 1-hexanol (Figure 3A), whereas the

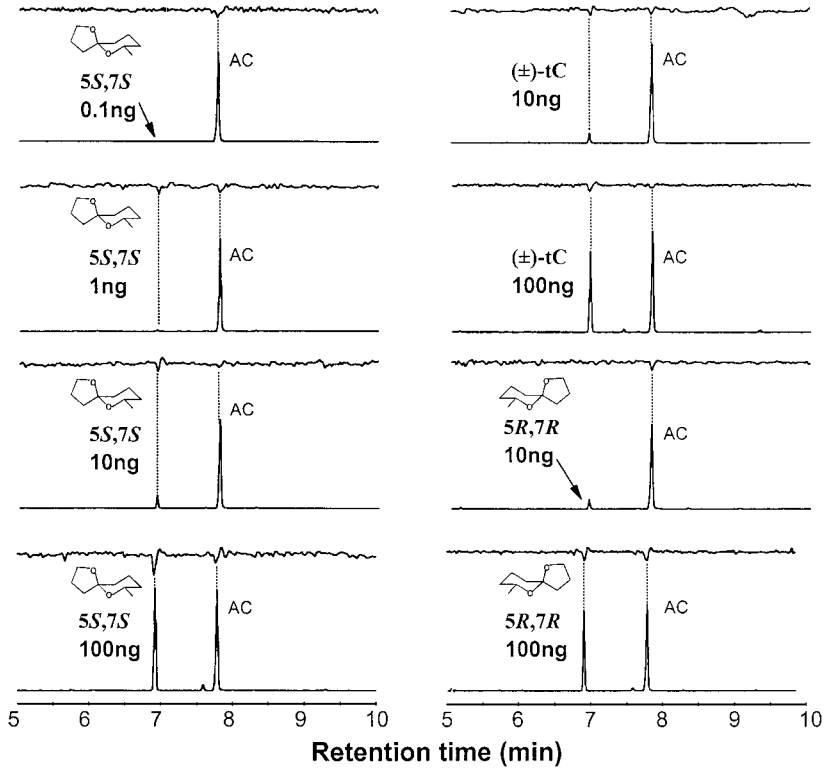


FIG. 2. Representative GC-EAD dose–response recordings on *Ips typographus* antennae to (5*S*,7*S*)-conophthorin, its antipode, and the racemate, (±)-tC. AC: active control, 100 ng of 1-hexanol.

(5*R*,7*R*)-isomer gave no responses. At the 10-ng level, again the racemate and the 5*S*,7*S*-enantiomer elicited strong EAD responses, which were stronger than that of 100 ng of 1-hexanol (Figure 3A), while the (5*R*,7*R*)-enantiomer was still inactive (Figures 2 and 3A). However, when the dosage went up to 100 ng, a clear EAD response to the (5*R*,7*R*)-sample was detected, with its response level similar to 100 ng of 1-hexanol (Figures 2 and 3A). Antennal responses to the (5*S*,7*S*)-enantiomer and the racemate were again much stronger than the active control (Figures 2 and 3A). An almost identical dose–response pattern as in *I. typographus* was found in *Ips duplicatus* (Figure 3B). Responses by *I. subelongatus* were also similar, but its relative responses to the (5*S*,7*S*)-enantiomer at levels of 1 to 100 ng seemed to be lower than those of the two other *Ips* species (Figure 3C). Additional tests on a pine bark beetle, *Orthotomicus erosus* (Woll.) (collected from naturally attacked *Pinus pinaster* Aiton trees, France) indicated that at the 10-ng level, the (5*R*,7*R*)-enantiomer was antennally inactive, while the (5*S*,7*S*)-enantiomer

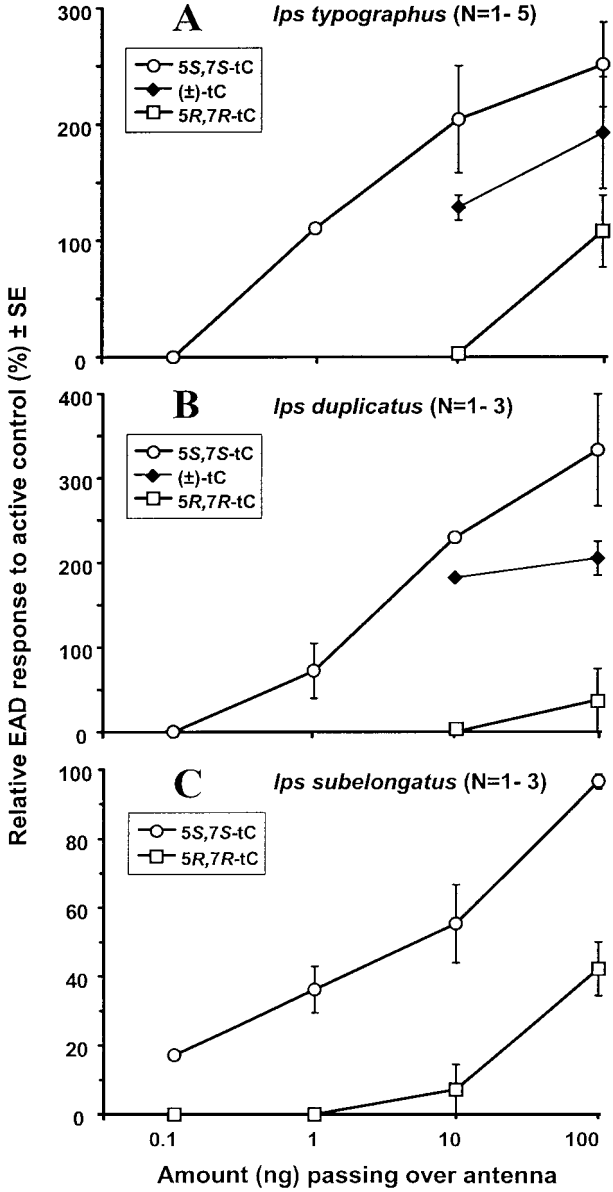


FIG. 3. EAD dose-responses by *Ips typographus* (A), *I. duplicatus* (B), and *I. subelongatus* (C) to different (*E*)-conophthorin enantiomers (5*S*,7*S*; 5*R*,7*R*) and the racemate, (±)-tC. The responses were normalized as the relative response (%) to active control (100 ng of 1-hexanol). For each dosage, and compound, 1–5 antennae were tested. (±)-tC was not tested on *I. subelongatus*.

elicited a strong EAD response similar to that caused by 100 ng of 1-hexanol (data not shown). There were no differences in the response patterns between males and females of these *Ips* species.

Using a similar GC-EAD technique, but equipped with a chiral column (coated with a modified cyclodextrin as the stationary phase), we tested (*E*)-conophthorin on males and females of a coniferophagous scolytid, *Dendroctonus micans*, and several angiosperm *Scolytus* species (*S. intricatus*, *S. mali*, *S. ratzeburgi*, *S. rugulosus*, and *S. scolytus*). Under the above experimental conditions, the enantiomeric separation showed an α value of 1.1 (retention time of the (5*S*,7*S*)-enantiomer divided by that of the (5*R*,7*R*)-enantiomer). While all antennae responded well to (5*S*,7*S*)-conophthorin, no reactions were recorded with its first eluting antipode, up to the 50-ng level (Figure 4A,B). In our investigations, antennae of *Hylesinus crenatus* and *Phloeophthorus rhododactylus* provided no answers [despite the fact that the beetles produce (*E*)-conophthorin], possibly due to experimental difficulties with antennal preparations.

DISCUSSION

Our results on GC-EAD dose–response indicate that only the (5*S*,7*S*)-enantiomer is detected by antennae of the three *Ips* species (probably *O. erosus* as well), with their response thresholds being between 0.1 and 1 ng, or lower, while the (5*R*,7*R*)-enantiomer is antennally inactive. The EAD responses caused by (5*R*,7*R*)-conophthorin at 100 ng (Figures 2 and 3A–C) could be explained by presence of ca. 1% of the (5*S*,7*S*)-enantiomer in the synthetic (5*R*,7*R*)-sample. The trace amount of this impurity (ca. 1 ng) would be sufficient to elicit an antennal response at the level similar to that caused by the active control (Figures 2 and 3). The enantiomeric selectivity of our chiral GC column was efficient enough to show the differential antennal activity of the two enantiomers of (*E*)-conophthorin (Figure 4).

Dose responses of insect antennae to semiochemicals have been widely studied by a stationary EAG technique [i.e., puffing volatiles over the antennal preparation (see Zhang, 2001 and references therein)], due to low costs, high throughput, and simplicity of handling. However, data concerning dosages used in EAG recordings usually represent the amounts of compounds loaded on the filter paper, which provides no information about the actual amounts of the tested compounds released during each puff. Moreover, EAG responses may depend on the experimental set up, and the activities of compounds with different volatilities can not be compared directly. However, using GC-EAD techniques, dosages of compounds with different volatilities can be easily controlled, and the amount of substances passing over the antenna1 preparations can be precisely determined. Furthermore, GC-EAD using a chiral column makes it possible to test the electrophysiological activity and response thresholds of highly pure

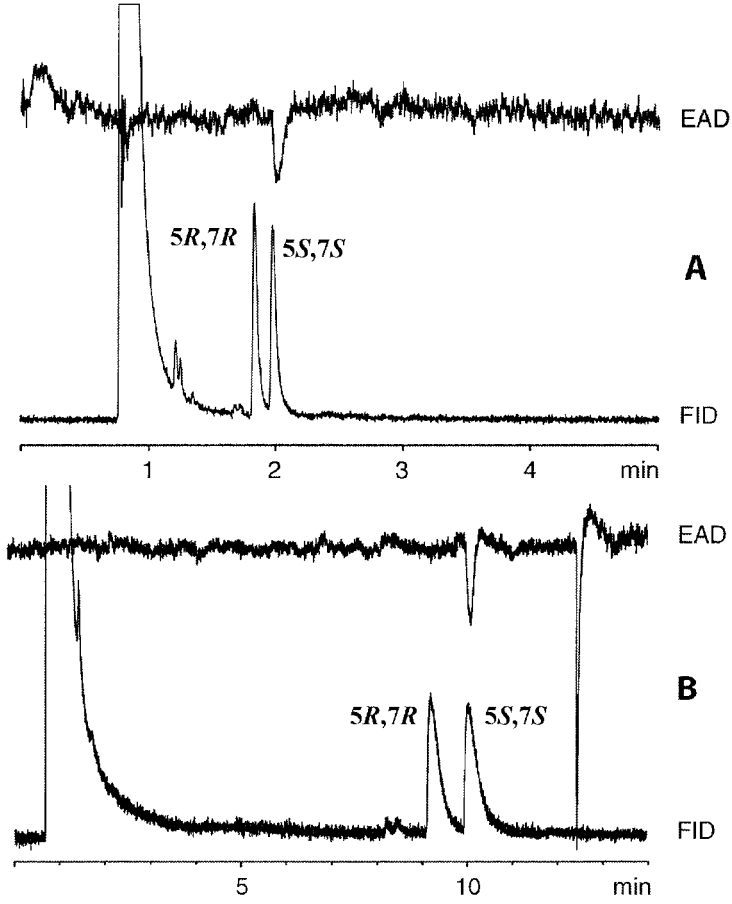


FIG. 4. (A) GC-EAD recording on *Dendroctonus micans* antenna. Response to the enantiomers of (*E*)-conophthorin separated upon chiral gas chromatography (conditions see Methods and Materials). (B) Representative GC-EAD recording on *Scolytus* antennae (here *S. ratzeburgi*). Response to the enantiomers of (*E*)-conophthorin separated upon chiral gas chromatography (conditions see Methods and Materials). Reactions of the other *Scolytus* spp. were similar. The sharp signal at 12.5 min is due to a puff of racemic (*E*)-conophthorin directly into the make-up airstream of the EAD system (not passing the GC system) to check whether the preparation is correctly functioning.

stereoisomers of chiral compounds without using synthetically obtained optically pure enantiomers (which may not always show an ee of 100%), as depicted in Figure 4.

Table 1 summarizes the current updated knowledge on the natural occurrence (production), and physiological as well as behavioral activity of conophthorin,

especially the *E* isomer. This spiroacetal appears to be widespread among several taxa of insects and plants. It may function as a male-produced repellent pheromone, a nonhost antiattractant synomone in bark beetles, and as an aggregation pheromone component in two twig beetle species (Table 1). The biological significance of the antennal reaction of the angiosperm bark beetles to (*E*)-conophthorin is largely unknown. However, in the ash bark beetle, *Leperisinus fraxini*, it acts as an intraspecifically active repellent (Kohnle et al., 1992). In most cases studied, only the naturally occurring and quantitatively dominant (5*S*,7*S*)-enantiomer was physiologically and behaviorally active, and no antagonistic or synergistic effects of the minor (5*R*,7*R*)-enantiomer were detected. The importance of the stereochemistry on the biological activity of chiral semiochemicals has been thoroughly reviewed by Mori (1998).

(*Z*)-Conophthorin also exists in nature (Table 1), but it is significantly less abundant and thermodynamically unstable. Due to lability, handling of pure enantiomers of (*Z*)-conophthorin is still impossible (W. Francke, unpublished results). Therefore, it does not seem to have any practical potential. It should be noted, however, that in many cases the naturally occurring conophthorin shows a lower *E*/*Z* ratio than a synthetic mixture prepared from the open chain ketodiol. A higher amount of the *Z*-isomer means that the original natural signal is thermodynamically unstable and may change the *E*/*Z* proportion with time and environmental conditions. Whether this phenomenon is of any biological significance, i.e., providing additional information for the receiver, e.g., about the age of the signal, awaits further investigation.

Evidence is increasing that (*E*)-conophthorin plays an important role in chemical ecology in general, and in host selection/nonhost avoidance in particular, of conifer bark beetles. It seems to be a good candidate to push conifer bark beetles away from their host trees. The latest investigations in Canada and Europe indicate that combinations of (*E*)-conophthorin with other NHVs and verbenone has shown potential for successful tree protection against mass attacks of *D. ponderosae* and *I. typographus* (Huber and Borden, 2001; Jakuš et al., 2002). Our findings on the enantiospecific antennal response to 5*S*7*S*-conophthorin by scolytid beetles, and the inactivity of its antipode, provide further support of the use of racemic (*E*)-conophthorin in future pest management programs against economically important bark beetles and other pest insects.

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MODULATION OF EXPLORATORY BEHAVIOR IN FEMALE MICE BY PROTEIN-BORNE MALE URINARY MOLECULES

CARLA MUCIGNAT-CARETTA*

Department of Human Anatomy and Physiology
University of Padova
Via Marzolo 3, 35131 Padova, Italy

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Abstract—Male pheromones are believed to attract females and repel male mice in open field tests but, when tested in more complex environments, they can attract male mice in usually avoided areas. Females were tested in an apparatus with one dark and one light side, in the absence or presence of male urine or the major urinary proteins (MUPs) bearing the natural ligands. Diestrous females were slower in leaving from the dark area when male urine or MUPs were present in it. Estrogen-primed females showed the opposite behavior, with an increase in the same latency. The light-avoidance behavior of prepubertal females, or females reared without males was not influenced by the presence of male chemosignals. The results show that adult female mice can react to MUP-borne volatiles as to adult male urine and use them as cues of male mice, if they were previously exposed to male cues during infancy. MUP-borne molecules are, thus, the olfactory trace of males in the environment and modulate mice exploratory behavior.

Key Words—Major urinary proteins, pheromones, urine, lipocalins, light-avoidance, mice.

INTRODUCTION

Male mice excrete several molecules in urine that are used as chemical cues to communicate their gender and social and reproductive status (Halpern, 1987). Urine is also used to mark the territory (Reynolds, 1971; Harrington, 1976). It represents a convenient aqueous medium that can be excreted in different amounts and in different situations (Hurst, 1990a,b). Male urine conveys chemical signals that act

* E-mail: carla.mucignat@unipd.it

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on both male and female mice, by modifying their behavior and modulating their hormonal status, with a direct involvement of the neuroendocrine hypothalamus (Keverne, 1983). Tests performed in simple environments showed that adult male urine attracts adult female mice and is submitted to prolonged investigation, while urine from castrated or prepubertal males is less attractive to females (Scott and Pfaff, 1970; Jones and Nowell, 1974). Dominant male urine attracts females the most (Hurst, 1990b; Mossman and Drickamer, 1996). The smell of adult male urine is attractive only to adult female mice, in fact prepubertal females prefer adult female chemosignals. The switch of female preference for adult male odor occurs at puberty (Coppola and O'Connell, 1988; Drickamer, 1989).

Different classes of molecules serve as chemical signals. Most are volatile molecules, but they can also be peptides. Chemical cues convey complex messages, such as those concerning sex and social status, possibly by combining different components. Mouse urine is rich in major urinary proteins (MUPs), which play a role in neuroendocrine modulation (Vandenbergh et al., 1975; Mucignat-Caretta et al., 1995a; Brennan et al., 1999; Marchlewska-Koj et al., 2000). MUP bind and carry small molecules as ligands in the urine, and act as pheromone carriers and reservoirs (Bocskei et al., 1992; Cavaggioni and Mucignat-Caretta, 2000). Some of the ligands are androgen-dependent molecules that act as male cues. The synthetic analogs of 2-*sec*-butylthiazoline and 2,3-dehydro-*exo*-brevicomin, two of the MUP volatile pheromones (Bacchini et al., 1992; Robertson et al., 1993), can concurrently induce aggressive behavior in male mice (Novotny et al., 1985). Moreover, the excretion of these two MUP ligands is androgen-dependent (Schwende et al., 1986), like the production of MUP (Clissold et al., 1984).

MUP-borne molecules have been shown to induce the same behavioral responses as intact male urine. In a simple preference test, they are avoided by adult males and preferred by adult females, while prepubertal mice do not display these preferences (Mucignat-Caretta et al., 1998). MUP-borne molecules can strongly influence male behavior in the presence of a receptive female by turning male sexual activity into aggressive behaviors (Mucignat-Caretta and Caretta, 1999a). These molecules can modify the light-avoidance behavior of male mice (Mucignat-Caretta and Caretta, 1999b), act as male cues, and elicit the same responses as intact male urine. If a mouse has to explore a new environment, which contains a well-illuminated area and a dark one, it shows a consistent behavior by choosing the dark side of the cage. The latencies in moving between the two compartments have been exploited to measure emotional reactivity in mice (Louilot et al., 1989) and have been used here to understand how (and which) pheromonal stimuli can modulate the behavior of mice in a relatively nonuniform environment.

Since responses to social odors are influenced by the hormonal status of the receiver, females in the diestrous phase of the estrous cycle were tested, measuring the escape latencies from one compartment to the other, first in the control condition without chemical stimuli, and then in the presence of male urine or MUPs on either

side of the apparatus. Since adult females modified their light-avoiding behavior in the presence of adult male urine in a different way from that already described for adult males (Mucignat-Caretta and Caretta, 1999b), it was relevant to look for an effect of MUP-borne volatiles that are as active in males as male urine. We tested whether MUPs purified from adult male urine in native conformation (with the ligands bound) modified the behavior of female mice as male urine does. We also tested for a differential response in estrogen-primed receptive female mice. This is an intriguing phenomenon, since it has been reported that adult females usually are attracted by male pheromonal cues in a simple environment, irrespective of the phase of their estrous cycle (Mossman and Drickamer, 1996). We also studied prepubertal females, since the responses to chemosignals are known to vary according to the age of the receiver, the preference of females for male odors becoming patent at puberty (Drickamer, 1989, Mucignat-Caretta et al., 1998). Finally, since the neuroendocrine responsivity to male pheromones is enhanced by previous exposure to male urine during infancy (Mucignat-Caretta et al., 1995b), adult diestrous females that were reared without fathers or any adult male were tested to see if they displayed the same responses to male pheromones as adult diestrous females reared with males.

METHODS AND MATERIALS

Subjects. The experiments were carried out according to Italian law on animal experiments and handling. Swiss mice (*Mus musculus*) were maintained at $23 \pm 1^{\circ}$C, on a 12L:12D photoregime with lights on at 6:00 AM. Mice were weaned at 21 days of age and then lived in groups (maximum six animals) of the same sex and age. Adult animals were tested at around 5 months of age. The estrous phase in adult females was checked by vaginal smears. In experiment 1, eleven females in the diestrous phase were tested in the presence or absence of adult male urine in the test cage. In experiment 2, seven females in the diestrous phase were tested with or without MUPs that were presented also to mice in experiments 3–5. In experiment 3, a group of seven estrogen-primed adult females was tested. All had a vaginal smear typical of estrus, characterized by cornified cells. Estrogen priming consisted of five daily injections of 17β-estradiol in oil (1 mg/kg body weight). Estrogen-primed females were chosen instead of naturally cycling females in order to reduce the variability, because estrus lasts only a few hours, and the hormonal peaks change widely among the early and late phases of estrus. Moreover, estrogen-primed females are a model of receptive females (Clark, 1993) and have already been used to test behavioral modifications induced by urinary chemical cues (Mucignat-Caretta and Caretta, 1999a). In experiment 4, a group of eight prepubertal females, 26 days old, was tested. In experiment 5, six females were tested. They were reared without adult males and had never come into contact

with adult males or their chemosignals. They were in the diestrous phase of their estrous cycle. All females in all experiments were virgins and naive with respect to sexual behavior. They had never come into contact with adult males prior to the test, except for their fathers during the preweaning period (for females tested in experiments 1–4). Male littermates were present in all litters up to weaning. In male mice during the first 21 days of life, there is no production or excretion of MUPs or MUP-associated androgen-dependent substances, as revealed by electrophoresis (unpublished observations) and gas chromatography (Mucignat-Caretta et al., 1995a).

Behavioral Assays. The behavioral test has been described previously (Mucignat-Caretta and Caretta, 1999b) and was based on a procedure sensitive to motivational changes (Crawley and Goodwin, 1980; Louilot et al., 1989; Misslin et al., 1989). The main innovation here consists of the introduction of pheromonal stimuli in to the test cage. The test apparatus consisted of a plastic cage divided into two compartments ($21 \times 27 \times 15$ cm each) by a plastic wall with a 4×3.5-cm opening. One (white) compartment had no cover to allow illumination (2000 lux) and behavioral recordings. The other (black) side was closed. Stimuli were put in five dishes (1.5 cm diam. $\times$ 0.5 cm high), four at the corners and one at the center of each compartment. A plastic mesh cap prevented contact with the chemical stimuli.

One hour before the test, mice were allowed free exploration (9 min) of the test cage in the absence of chemical signals. The test began when the mouse was put into the dark compartment. The first measurement (dark-to-light time, DL1) was the time elapsed from this moment to the complete crossing of the door (all four paws in the light compartment). The mouse was then put briefly in its home cage, to allow cleaning of the test cage if needed, and subsequently released in the light compartment. Thereafter, the time spent in the light compartment up to the complete crossing of the door toward the dark side (light-to-dark time, LD) was measured. The mouse could then return freely to the light compartment: the time spent in the dark compartment up to the crossing of the door to the light compartment was the second dark-to-light time (DL2). The maximum time allowed for each measure was 3 min. The control test was performed without chemical stimuli in the apparatus. The other two conditions consisted of putting a fixed amount (100 μl) of adult male urine or MUP (20 mg/ml solution) in each well, either in the dark (condition: MUPs on dark) or in the light compartment (condition: MUPs on light). The cage was cleaned, and the number of urine spots, possibly related to countermarking, and fecal pellets, related to autonomic activation, was counted after DL1 was recorded at the end of each test (training period; control–no chemical stimuli; stimuli on light compartment; stimuli on dark compartment).

Statistical Analysis. The data on latencies were analyzed separately for each experiment, with a two-way ANOVA, with the factors condition (control, urine, MUPs on light, and urine or MUPs on dark) and Test (DL1, LD, DL2). Post-hoc

Newman-Keuls comparisons were conducted whenever appropriate. The order of administration of trials was counterbalanced for the mice.

Biochemical Procedures. MUPs were purified according to Mucignat-Caretta and Caretta, 1999b. Urine from a pool of 12–20 adult male mice (3–4 month old) was collected overnight and frozen. After thawing, it was used directly or to purify MUPs, and then centrifuged (10,000 g, 30 min). The supernatant was filtered onto paper, then concentrated through an ultrafiltration cell (Amicon YM10, 10,000 Da cutoff) under nitrogen pressure. The eluate was chromatographed through a G-50 (Pharmacia) molecular sieve column (1.5 cm ID, 60 cm long, 4 ml/hr flow), and samples (5 ml each), were collected. Samples containing MUPs were rechromatographed through the G-50 column. The MUP-containing fractions were pooled and reconcentrated as above. The purified stimuli were analyzed by denaturing gel electrophoresis, where a single band of apparent molecular mass around 18–19 kDa could be detected, as well as by gas chromatography, where the typical profile of MUP ligands (Mucignat-Caretta et al., 1995a; Marchlewska-Koj et al., 2000) could be observed.

RESULTS

Mean latencies +SEM for all experiments are shown in Figure 1. Table 1 reports the number of mice urinating and defecating in each period and the respective number of urine drops and fecal pellets in each of the two compartments. Since only a small number of mice showed either of these responses, the data were not further analyzed.

Experiment 1 on diestrous females tested with male urine showed that the factor Test was significant, $F_{2,20} = 44.987$, $P < 0.001$, with LD faster than both DL1 and DL2. DL2 also was different from DL1, a result best explained by the significant interaction Test × Condition, ($F_{4,40} = 6.428$, $P < 0.001$) that showed that for control and urine on light condition, DL1 and DL2 are not different, while for condition urine on dark, DL2 is significantly shorter than DL1.

The ANOVA on data from diestrous females tested with MUPs (experiment 2) revealed that the factor Test reached significance ($F_{2,12} = 21.284$, $P < 0.001$), with LD faster than DL1 and DL2. The interaction Test × Condition also was significant ($F_{4,24} = 3.089$, $P < 0.05$) as in experiment 1; for control and MUPs on light conditions, DL1 and DL2 do not differ, while for condition MUPs on dark, DL2 is shorter than DL1.

The analysis on estrogen-primed females tested with MUPs (experiment 3) showed that the factor Test was significant ($F_{2,12} = 11.008$, $P < 0.01$), with LD faster than the other two latencies. The interaction Test × Condition also was significant ($F_{4,24} = 3.108$, $P < 0.05$). The post-hoc test showed that while for the control and MUPs on light conditions the latencies in the three tests do not differ,

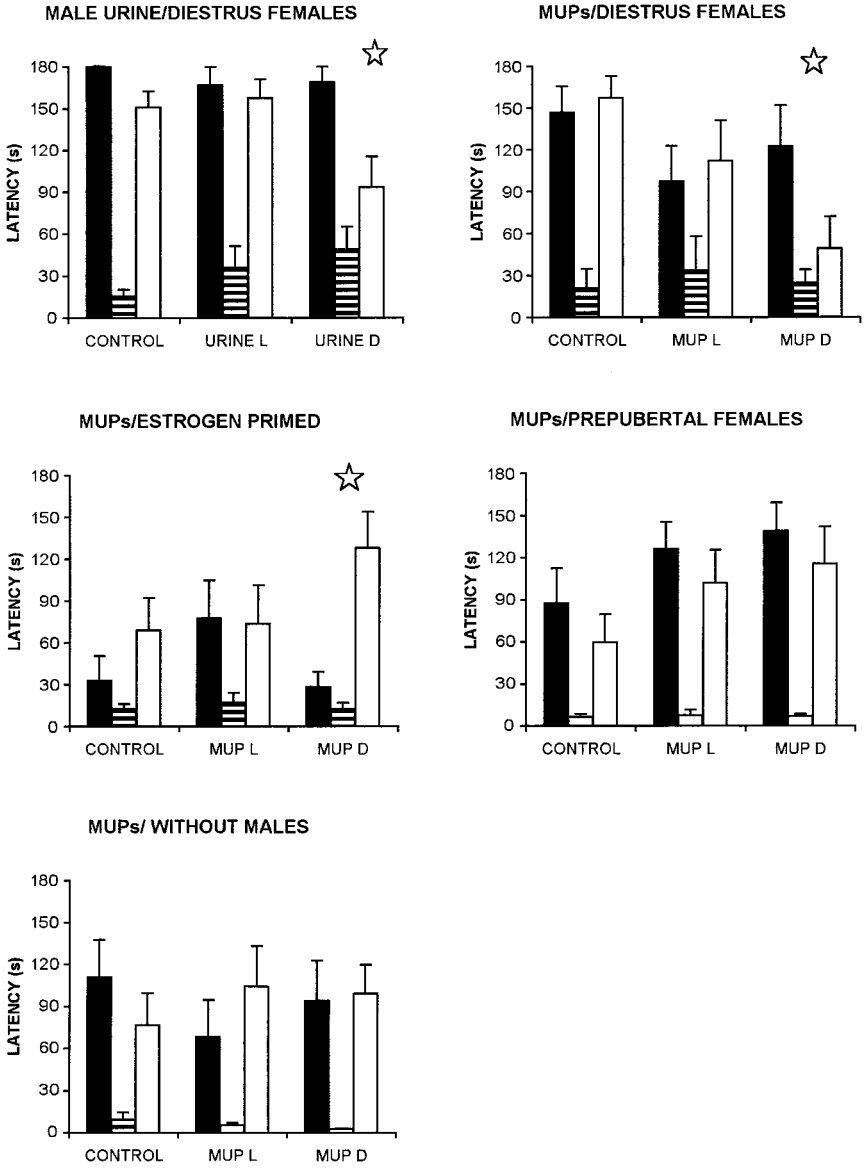


FIG. 1. The effect of male chemical cues on the light-avoidance behavior of different groups of female mice. Filled bars refer to DL1 (the first dark-to-light) latency, shaded bars to LD (light-to-dark) latency, and open bars to DL2 (the second dark-to-light) latency. Means + SEM are shown. The following conditions were tested: control: no chemical stimuli in the environment; urine (or MUP) L: stimuli on the light compartment; urine (or MUP) D: stimuli on the dark compartment. $^{*}P < 0.05$, DL2 different from DL1.

TABLE 1. EXPERIMENTAL RESULTS[a]

	Urine		Feces	
	Mice (N)	Urine spots (N)	Mice (N)	Pellets (N)
Experiment 1: 11 diestrous females				
Training	0	0	0	0
Control	0	0	4	5D
Urine L	0	0	4	7D, 2L
Urine D	1	1D	4	6D, 2L
Experiment 2: 7 diestrous females				
Training	0	0	1	2D
Control	1	1D	2	6D
MUP L	1	1L	3	6D
MUP D	2	2D	2	2D
Experiment 3: 7 estrogen-primed females				
Training	0	0	0	0
Control	0	0	2	3D
MUP L	3	3D, 2L	2	3D, 2L
MUP D	6	6D, 1L	3	5D, 1L
Experiment 4: 8 prepubertal females				
Training	0	0	0	0
Control	0	0	0	0
MUP L	0	0	0	0
MUP D	0	0	0	0
Experiment 5: 6 females reared without males				
Training	0	0	1	1D
Control	0	0	2	5D
MUP L	0	0	0	0
MUP D	0	0	3	5D

[a] At the end of each test (indicated in the first column) the following data were obtained: the total number of mice that urinated, the number of urine drops counted in each compartment; the number of mice that defecated and the number of fecal pellets in each compartment (D: dark; L: light). In the first column, the following conditions are indicated: training period, control (no chemical stimuli), chemical stimuli (male urine or MUP) on light compartment, stimuli on dark compartment.

for the condition MUPs on dark, DL2 is longer than DL1 and LD. The absolute values of the latencies for the control condition in estrogen-primed females are smaller than in all other groups of mice that have been tested. This can be related to an increased reactivity already described as a consequence of estrogen priming (Rodier, 1971).

The ANOVA on prepubertal females (experiment 4) showed only a significant effect on Test ($F_{2,14} = 28.3750$, $P < 0.001$) with LD shorter than the other latencies (DL1 and DL2). On the other hand, the ANOVA on females reared without adult males (experiment 5) showed that the only significant factor was Test ($F_{2,10} = 14.049$, $P < 0.002$) with LD shorter than DL1 and DL2.

DISCUSSION

Stimuli acting through the olfactory systems modify a variety of physiological and behavioral responses ranging from puberty onset and blocking pregnancy (Keverne, 1983; Tirindelli et al., 1998), to aggressive behavior and urine marking (Maruniak et al., 1986). The present data address the question of the impact of specific fractions of male urine on exploratory behavior in female mice of different ages and hormonal status. The dark–light emergence test is based on the conflict between the need to avoid brightly illuminated areas and the tendency to explore new environments. This conflict results in mice spending more time in the dark chamber (Crawley and Goodwin, 1980; Misslin et al., 1989). The present findings confirm that light avoidance behavior is present in all groups of mice that have been tested and provide some information on how female mice cope with relevant stimuli coming from different sensory modalities. All groups of mice in the control condition display a preference for the dark compartment over the illuminated one; they go faster from the light compartment to the dark one (LD) than the reverse, as expected. When present, an effect is apparent on DL2 latency, when the mouse has to freely return to the light compartment, after having experienced both the dark and the light side. The effect of chemosignals only on DL2 measurements was also apparent in males (Mucignat-Caretta and Caretta, 1999b). This is possibly related to the fact that the mouse has already experienced both sides of the cage, and, thus, can choose its preferred side.

Adult diestrous females in the control condition show a clear preference for the dark compartment, as demonstrated by longer latencies to escape from the dark compartment (DL1 and DL2) compared to the latency to enter in the dark compartment (LD). As expected, no difference can be discerned between the first and the second latency to escape from the dark side (DL1 and DL2). When adult male urine or MUPs are present in the dark compartment, a significant reduction in DL2 time is observed. This behavior suggests that the chemical cues borne by MUPs are a sufficient stimulus for the mouse to behave as in the presence of male urine, and hence to escape from the usually preferred dark side.

Stud males present the opposite tendency: they enter the light side faster when MUPs or male urine are present in it (Mucignat-Caretta and Caretta, 1999b). It is reasonable to speculate that nonreceptive females prefer to avoid an environment in which the presence of males is evident, while male mice tend to explore it, possibly in relation to a countermarking behavior (Hurst, 1990a). On the other hand, receptive females in the same condition (MUPs on dark) show a significant increase of DL2. This result can be described as the preference of estrous females for MUPs-associated chemical cues that attract them. In contrast, despite having already experienced male cues from their fathers, prepubertal females seem not to be influenced in any way by male cues and display light-avoidance behavior irrespective of male cues. These results confirm that the responsivity to male urinary

chemosignals develops at puberty. The data from females reared in the absence of adult males indicates that, as for other pheromonal effects (Mucignat-Caretta et al, 1995b), the behavioral responsivity to male chemosignals is modulated by the experience of stimuli during the early phases of development.

Autonomic activation within each group, measured as the number of mice that defecated, did not seem to vary in the different conditions. Moreover, urination acts were not frequent; only estrogen-primed females urinated more when chemical stimuli were present in the apparatus, and this result, if confirmed by further studies, would support a role for these stimuli as meaningful indicators of sexual identity.

Overall, the results of the present experiments indicate that MUP-borne molecules elicit different responses in groups of females of different age and hormone status. It can be concluded that MUP-borne molecules act as male cues since they are avoided by diestrous females as much as male urine and are attractive to estrous females. This effect of male cues is evident after puberty, as shown in experiment 4, and is influenced by the previous experience of the females (experiment 5).

The present data help to clarify the mechanism of coding the sexual identity of the emitter: in this respect, the volatile molecules bound by MUPs are sufficient to act as male chemosignals, so they are the minimal information that signals the presence of a male mouse. Consistent with this hypothesis, is the combination of environmental conditions and chemical signals that elicits differential responses in the different groups of mice. This behavior can be more differentiated compared to that seen in less complex tests. For example, there is no difference in the behavior observed in estrous and nonestrous females towards urinary stimuli presented in a uniform environment (Mossman and Drickamer, 1996). The present experiments discriminate more subtle differences in behavior: nonestrous females avoiding male cues and receptive females preferring them. This is not surprising, since gonadal steroid hormones modulate the activity in different areas of the brain, including those involved in chemical perception and sexual behavior. Some of the sexually dimorphic nuclei in the brain pertain to the main or the accessory olfactory pathways, like the nuclei of the medial amygdala and medial preoptic hypothalamus (Brennan and Keverne, 1997); they express gonadal steroid receptors, and their activity is modulated by circulating steroid hormones. During the estrous phase both the expression of the neuropeptide cholecystokinin and the electrical activity in these nuclei (Simerly, 1990; Li et al., 1992) are characterized by maximal sensitivity to pheromonal stimuli.

In conclusion, the present data show that urinary chemical cues, and in particular MUPs bearing ligands, modify the light-avoidance behavior of female mice, depending on the age and endocrine status of the receiver. The system composed of MUPs and ligands is, thus, perceived as a complex signal that mediates different pheromonal effects, both endocrine (Mucignat-Caretta et al., 1995) and behavioral.

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VOLATILE COMPONENTS IN DEFENSIVE SPRAY OF THE HOODED SKUNK, *Mephitis macroura*

WILLIAM F. WOOD,[1,*] BRIAN G. SOLLERS,[1] GWEN A. DRAGOO,[2] and JERRY W. DRAGOO[3]

[1]*Department of Chemistry*
Humboldt State University
Arcata, California 95521, USA

[2]*Albuquerque Biological Park*
903 Tenth Street SW
Albuquerque, New Mexico 87102, USA

[3]*Department of Biology*
University of New Mexico
Albuquerque, New Mexico 87131, USA

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Abstract—GC-MS analysis of the anal sac secretion from the hooded skunk, *Mephitis macroura*, showed the following seven major components comprised 99% of the volatiles in this secretion: (*E*)-2-butene-1-thiol, 3-methyl-1-butanethiol, *S*-(*E*)-2-butenyl thioacetate, *S*-3-methylbutenyl thioacetate, 2-phenylethanethiol, 2-methylquinoline, and 2-quinolinemethanethiol. Minor volatile components identified in this secretion are phenylmethanethiol, *S*-phenylmethyl thioacetate, *S*-2-phenylethyl thioacetate, bis[(*E*)-2-butenyl] disulfide, (*E*)-2-butenyl 3-methylbutyl disulfide, bis(3-methylbutyl) disulfide, and *S*-2-quinolinemethyl thioacetate. This secretion is similar to that of the striped skunk, *Mephitis mephitis*, differing only in that it contains four compounds not reported from the striped skunk: phenylmethanethiol, *S*-phenylmethyl thioacetate, 2-phenylethanethiol, and *S*-2-phenylethyl thioacetate.

Key Words—Mephitidae, *Mephitis macroura*, hooded skunk, anal sac secretion, (*E*)-2-butene-1-thiol, 3-methyl-1-butanethiol, *S*-(*E*)-2-butenyl thioacetate, *S*-3-methylbutenyl thioacetate, 2-phenylethanethiol, 2-methylquinoline, 2-quin-olinemethanethiol.

* To whom correspondence should be addressed. E-mail: wfw2@humboldt.edu

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INTRODUCTION

The defensive secretions sprayed from the anal sac of skunks are well known for their malodorous nature. The striped skunk has been the subject of many chemical investigations (Swarts, 1862; Löw, 1879; Aldrich, 1896; Aldrich and Jones, 1897; Stevens, 1945; Andersen and Bernstein, 1975; Andersen et al., 1982; Wood, 1990). The volatile components in the spray of two other North American skunks, the spotted skunk (Wood et al., 1991) and the hog-nosed skunk (Wood et al., 1993) have also been identified. In the present study, we report the volatile components of the anal sac from the hooded skunk, *Mephitis macroura*.

METHODS AND MATERIALS

The defensive secretion from a single male hooded skunk from Hidalgo County, New Mexico, USA, was examined. A sample of the secretion was obtained from each anal sac of the anaesthetized animal by inserting a needle into the sac and withdrawing the contents. The secretion was placed in a screw-cap vial, and shipped at ambient temperature to Arcata, California, USA. Within 3 days of collection, a 20-mg portion of this secretion from each gland was placed in 1.5 ml of dichloromethane and analyzed by gas chromatography–mass spectrometry (GC-MS).

The GC-MS analyses were performed on the dichloromethane extracts in a splitless mode (0.5 min), using a Hewlett-Packard GCD Plus fitted with a 30-m $\times$ 0.25-mm cross-linked phenyl methyl silicone capillary column (HP-5MS). The gas chromatograph was programmed so the oven temperature was kept at 40°C for 4 min, then increased to a final temperature of 325°C at a rate of 30°C/min and kept at this temperature for 2 min. Mass spectral fragments below $m/z = 39$ were not recorded. The relative amount of each component was determined as the percent of the total ion current (TIC).

Identification of the components was done by comparison of mass spectra and gas chromatographic retention times to reference compounds. (*E*)-2-Butene-1-thiol, *S*-(*E*)-2-butenyl thioacetate, *S*-3-methylbutenyl thioacetate, 2-quinolinemethanethiol, and *S*-2-quinolinemethyl thioacetate were obtained from striped skunk secretion (Andersen and Bernstein, 1975; Wood, 1990). 3-Methyl-1-butanethiol, 2-phenylmethanethiol, and 2-methylquinoline were purchased from Aldrich Chemical Co. 2-Phenylethanethiol was prepared by the method of Urquhart et al. (1955) from (2-bromoethyl)benzene. *S*-phenylmethyl thioacetate and *S*-2-phenylethyl thioacetate by reacting phenylmethanthiol or 2-phenylethanethiol with acetyl chloride. The mass spectrum of *S*-phenylmethyl thioacetate is (EI-MS): $m/z = 166$ (M^+, 19), 124(9), 123(26), 122(20), 121(5), 92 (8), 91(100), 89(5), 77(9), 64(17), 63(7), 51(9), 50(4), 45(20), and 43(72), and that of *S*-2-phenylethyl thioacetate is (EI-MS): $m/z = 180$ (M^+, 3), 135(3), 105(12), 104(100), 103(3), 92 (5), 91(32),

79(3), 78(5), 77(9), 65(9), 63(3), 51(6), 43(44), and 39(4). Bis[(*E*)-2-butenyl] disulfide, (*E*)-2-butenyl 3-methylbutyl disulfide, and bis(3-methylbutyl) disulfide were prepared by treatment of the defensive secretion with iodine as described by Wood (1990). Mass spectra have previously been reported for (*E*)-2-butene-1-thiol, 3-methyl-1-butanethiol, *S*-(*E*)-2-butenyl thioacetate, *S*-3-methylbutenyl thioacetate, 2-methylquinoline, 2-quinolinemethanethiol, bis[(*E*)-2-butenyl] disulfide, (*E*)-2-butenyl 3-methylbutyl disulfide, and bis(3-methylbutyl) disulfide by Wood (1990); for 2-phenylethanethiol by Wood et al. (1991); and for phenylmethanethiol in the NIST 1998 computerized mass spectral library.

RESULTS AND DISCUSSION

The GC-MS analyses on two samples of hooded skunk anal sac secretion are reported in Table 1. The variation in composition between the two anal sacs was small and disappeared on rounding to two figures. The analyses showed seven major components at a concentration greater than 1% to make up 99% of the volatiles (Figure 1). These compounds and their retention times (R_t), are (*E*)-2-butene-1-thiol (**1**, $R_t = 3.06$ min), 3-methyl-1-butanethiol (**2**, $R_t = 4.19$ min),

TABLE 1. COMPOSITION OF MAJOR VOLATILE COMPONENTS OF ANAL SAC SECRETION FROM FOUR SPECIES OF NORTH AMERICAN SKUNKS[a]

Compound	Amount (%)			
	Hooded skunk[b]	Striped skunk[c]	Spotted skunk	Hog-nosed skunk
(*E*)-2-Butene-1-thiol	32	38–40	30–36	71
3-Methyl-1-butanethiol	39	18–26	48–66	
S-(*E*)-2-Butenyl thioacetate	16	12–18		17
S-3-Methylbutanyl thioacetate	7	2–3		
Phenylmethanethiol	0.3		0.2–0.8	1
2-Phenylethanethiol	1.4		2–5	
2-Methylquinoline	2.3	4–11	0.3–0.9	2
Bis[(*E*)-2-butenyl] disulfide	trace		trace	3
S-Phenylmethyl thioacetate	trace			
(*E*)-2-Butenyl 3-methylbutyl disulfide	0.5	0.2–1.6	0.2–0.6	
Bis(3-methylbutyl) disulfide	trace		0.1–0.2	
S-2-Phenylethyl thioacetate	0.2			
2-Quinolinemethanethiol	1.3	4–12	0.2–0.3	0.5
S-2-Quinolinemethyl thioacetate	trace	1–4		

[a] In order of increasing retention times. Data from Wood (1990) and Wood et al. (1991, 1993).
[b] Variation of composition for the two anal sacs was small and disappeared on rounding to two figures.
[c] Compounds less than 1% were not identified in this study.

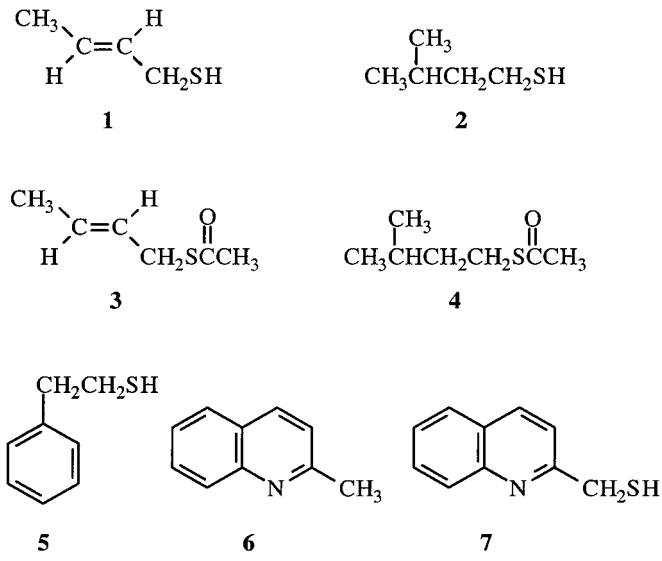


FIG. 1. Major components form *Mephitis macroura* defensive secretion: (*E*)-2-butene-1-thiol (**1**), 3-methyl-1-butanethiol (**2**), *S*-(*E*)-2-butenyl thioacetate (**3**), *S*-3-methylbutenyl thioacetate (**4**), 2-phenylethanethiol (**5**), 2-methylquinoline (**6**), and 2-quinolinemethanethiol (**7**).

S-(*E*)-2-butenyl thioacetate (**3**, $R_t = 6.77$ min), *S*-3-methylbutenyl thioacetate (**4**, $R_t = 7.12$ min), 2-phenylethanethiol (**5**, $R_t = 8.08$ min), 2-methylquinoline (**6**, $R_t = 8.77$ min), and 2-quinolinemethanethiol (**7**, $R_t = 10.24$ min).

Seven minor components were identified from this secretion (Figure 2). These compounds and their retention times (R_t) are phenylmethanethiol (**8**, $R_t =$ 7.49 min), bis[(*E*)-2-butenyl] disulfide (**11**, $R_t = 8.81$ min), *S*-phenylmethyl thioacetate (**9**, $R_t = 8.94$ min), (*E*)-2-butenyl 3-methylbutyl disulfide (**12**, $R_t =$ 9.00 min), bis(3-methylbutyl) disulfide (**13**, $R_t = 9.23$ min), *S*-2-phenylethyl thioacetate (**10**, $R_t = 9.33$ min), and *S*-2-quinolinemethyl thioacetate (**14**, $R_t =$ 11.23 min). This is the first report of *S*-phenylmethyl thioacetate and *S*-2-phenylethyl thioacetate as natural products. All other compounds have previously been identified in skunk spray (Andersen and Bernstein, 1975; Wood, 1990; Wood et al., 1991, 1993).

Table 1 compares the defensive secretion of four skunk species. The defensive spray of the hooded skunk is similar to that of the striped skunk, as reported by Wood (1990). If differs by having four additional compounds that were not reported from the striped skunk. These are phenylmethanethiol, 2-phenylethanethiol, *S*-phenylmethyl thioacetate, and *S*-2-phenylethyl thioacetate. 2-Phenylethanethiol has been reported from spotted skunk defensive secretion (Wood et al., 1991) but

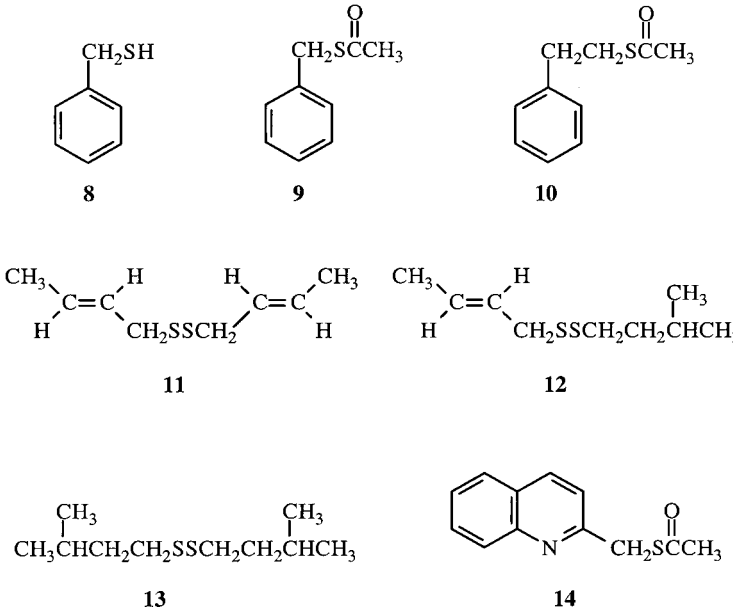


FIG. 2. Minor components identified in *Mephitis macroura* defensive secretion: phenylmethanethiol (**8**), *S*-phenylmethyl thioacetate (**9**), *S*-2-phenylethyl thioacetate (**10**), bis[(*E*)-2-butenyl] disulfide (**11**), (*E*)-2-butenyl 3-methylbutyl disulfide (**12**), bis(3-methylbutyl) disulfide (**13**), and *S*-2-quinolinemethyl thioacetate (**14**).

not from the hog-nosed skunk (Wood et al., 1993). The relative amounts of the two most abundant compounds are also different. In the hooded skunk, 3-methyl-1-butanethiol is the major compound, while (*E*)-2-butene-1-thiol is more abundant in the striped skunk.

The hooded skunk, striped skunk, and the hog-nosed skunk have a thioacetate derivative for each of the free thiols in their secretion. Perhaps the components in these skunks' defensive spray are biosynthesized as the thioacetates and then hydrolyzed to the thiol, as thioacetates are used in many biosynthetic pathways. These thioacetates may make the effects of this secretion longer lasting. The thioacetate derivatives can hydrolyze slowly and continue to release the offensive-smelling thiols over a period of time. Anecdotal reports about pets sprayed by striped skunks, indicate that skunk odor later returns to sprayed animals when their hair is wet.

Several DNA studies have been done on the relatedness of the three skunk genera: striped and hooded skunks (*Mephitis*), spotted skunks (*Spilogale*), and hog-nosed skunks (*Conepatus*). These studies indicate that skunks of the genus *Mephitis* and *Spilogale* may be more closely related to each other than either is to the genus *Conepatus* (Dragoo et al., 1993; Dragoo and Honeycutt, 1997). Variations

in the defensive spray of these three genera were examined to see if they support this relationship (Table 1). Of the major free thiols, *Mephitis* secretion has (*E*)-2-butenethiol, methylbutanethiol, and phenylethanethiol as does *Spilogale*, while *Conepatus* has only (*E*)-2-butenethiol. On this basis, *Mephitis* and *Spilogale* may seem more closely related. However, *Mephitis* and *Conepatus* have thioacetate derivatives of all of the free thiols, types of compounds that are missing from *Spilogale*. On this basis, *Mephitis* and *Conepatus* seem to be more closely related. Thus, examination of presently known secretion components can neither confirm nor refute genetic studies, but if the secretions from other *Conepatus* spp. are different, they could support these studies.

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CONTROL OF WITCHWEED *Striga hermonthica* BY INTERCROPPING WITH *Desmodium* spp., AND THE MECHANISM DEFINED AS ALLELOPATHIC

ZEYAUR R. KHAN,[1] AHMED HASSANALI,[2] WILLIAM OVERHOLT,[2] TSANUO M. KHAMIS,[3] ANTONY M. HOOPER,[4] JOHN A. PICKETT,[4,*] LESTER J. WADHAMS,[4] and CHRISTINE M. WOODCOCK[4]

[1]*The International Centre of Insect Physiology and Ecology*
Mbita Point Field Station
P.O. Box 30, Mbita, S. Nyanza, Kenya

[2]*The International Centre of Insect Physiology and Ecology*
P.O. Box 30772, Nairobi, Kenya

[3]*Chemistry Department*
Jomo Kenyatta University of Agriculture and Technology
P.O. Box 62000, Nairobi, Kenya

[4]*Biological and Ecological Chemistry Department*
IACR-Rothamsted
Harpenden, Hertfordshire AL5 2JQ, United Kingdom

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Abstract—During investigations into the control of insect damage to maize crops in subsistence farming in Kenya, which involved intercropping with repellent plants, the fodder legumes silverleaf (*Desmodium uncinatum*) and greenleaf (*D. intortum*) were also found to reduce dramatically the infestation of maize by parasitic witchweeds such as *Striga hermonthica*. This effect was confirmed by further field testing and shown to be significantly greater than that observed with other legumes, e.g., cowpea, as were the concomitant yield increases. The mechanism was investigated, and although soil shading and addition of nitrogen fertilizer showed some benefits against *S. hermonthica* infestation, a putative allelopathic mechanism for *D. uncinatum* was observed. In screenhouse studies, a highly significant reduction in *S. hermonthica* infestation was obtained when an aqueous solution, eluting from pots in which *D. uncinatum* plants were growing, was used to irrigate pots of maize planted in soil seeded with high levels of *S. hermonthica*. Growth of the parasitic weed was almost completely suppressed, whereas extensive infestation occurred with the control eluate. Laboratory investigations into the allelopathic effect of *D. uncinatum*, using samples of water-soluble chemical components exuded from cleaned roots, demonstrated that this

* To whom correspondence should be addressed. E-mail: john.pickett@bbsrc.ac.uk

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involved a germination stimulant for *S. hermonthica* and also an inhibitor for haustorial development.

Key Words—Allelopathy, maize, *Striga*, *Desmodium*, nitrogen, shading, silverleaf, germination stimulant, suicidal germination.

INTRODUCTION

The witchweeds are parasitic plants that, after germination, attach to the roots and cause considerable interference with normal development of their host plants (Parker and Riches, 1993). Witchweeds threaten the staple food of over 100 million people in Africa (M'boob, 1989; Musselman et al., 1991; Lagoke et al., 1991). Of the 23 species prevalent in Africa, *Striga hermonthica* (Scrophulariaceae) is the most significant, parasitizing a wide range of crops including maize, sorghum, millet, fonio, rice, and sugar cane (Musselman, 1994; Berner et al., 1996). Other witchweed species of importance in Africa are *S. aspera*, *S. asiatica*, and *S. passargei* on cereals, *S. gesnerioides* on cowpea, and *Alectra vogelli* (Scrophulariaceae) on cowpea, groundnut, and soybean. Although *S. aspera* originally parasitised wild grasses, it now has an extended host range that includes maize (Carson, 1989). Seeds can remain viable in the soil for up to 20 years and can be spread by both mechanical and cultural processes. This study focuses on *S. hermonthica*, for which there are currently no viable control methods suitable for the socioeconomic conditions of the most afflicted regions in Africa (Lagoke et al., 1991; Berner et al., 1995).

Control of *S. hermonthica* was observed during the development of a control strategy against stem borers in maize in eastern Africa. The strategy involved creating a push–pull effect using highly attractive trap crops to aggregate oviposition of adult stem borers, for example, *Busseola fusca* (Noctuidae) and *Chilo partellus* (Pyralidae), away from the central maize stand, and intercropping between the maize rows with repellent plants (Khan et al., 2000). The most effective of the repellent intercrops was molasses grass, *Melinis minutiflora*, and this plant additionally caused an increase in parasitization of those stem borers that were not aggregated away from, or directly repelled from, the maize (Khan et al., 1997). However, the legumes silverleaf, *Desmodium uncinatum*, and greenleaf, *D. intortum*, were also investigated as intercrops because of their high value as cattle forage and for their role in fixing atmospheric nitrogen. In trials in the Suba district of Kenya, where *S. hermonthica* is highly prevalent, these legumes were found to give an unexpected and dramatic reduction in infestation by this witchweed.

The objective of the current study was to compare directly the effect of *Desmodium* species with that of other plants reported to reduce colonization by witchweeds (Carson, 1986) and to investigate the mechanism by which control of the parasitic plant is effected. This could be by increasing available nitrogen, by

offering shade, or by an allelopathic effect involving chemicals associated with the roots, all of which are mechanisms known to give some control of these parasitic weeds (Press and Gurney, 2000).

METHODS AND MATERIALS

Seeds. Seeds of the following plants were employed in these studies: silverleaf, *Desmodium uncinatum* (Jacq.) DC. (Fabaceae), greenleaf, *Desmodium intortum* (Mill.) Urb. (Fabaceae), maize, *Zea mays* L. (Poaceae) (var. Hybrid 513), African witchweed, *Striga hermonthica* (Del.) Benth. (Scrophulariaceae), soybean, *Glycine max* (L.) (Fabaceae) (var. EAI 3600), sun hemp, *Crotolaria juncea* L. (Fabaceae), and cowpea, *Vigna unguiculata* (L.) (Fabaceae) (var. ICV 2).

Field Trials. Field trials were conducted at ICIPE's Mbita Point Field Station in the Suba District of Kenya, on the shores of Lake Victoria, where *S. hermonthica* poses a serious constraint to the cultivation of cereals. Field plots with at least 100 *S. hermonthica* seeds/250 g soil were used for all experimental trials.

To evaluate the effects of different legumes on the reduction of witchweed infestation, maize was intercropped in alternate rows with one of five species of legumes: cowpea, soybean, sun hemp, greenleaf, or silverleaf. Maize was planted with a row-to-row distance of 75 cm and a plant-to-plant distance of 25 cm within a row. A control plot of a maize monocrop was also included. The six treatments were randomized and planted in six replications in a 6×6 quasicomplete Latin square design (Baily, 1984; Smart et al., 1994). Each treatment plot was 6 m $\times$ 6 m. The maize used was a witchweed-susceptible, medium maturity commercial variety, Hybrid 513, recommended for mid-altitude regions with moderate rainfall. The trials were planted during the 1998 long rainy season (March to July). Infestation levels were assessed nondestructively by selecting, at random, 63 maize plants in each treatment plot and counting the number of emerged *S. hermonthica* plants present. Crop yields were evaluated by harvesting a 5-m $\times$ 5-m block in the center of each plot, leaving a 1-m unharvested border to avoid edge effects.

To investigate the role of shading, nitrogen, and allelopathy in witchweed suppression by *D. uncinatum*, field trials were established during the long rainy season of 1999 and the short rainy season of 1999–2000. Field plots, 6 m $\times$ 6 m, were arranged in a six-block quasicomplete design (Baily, 1984; Smart et al., 1994). Each treatment appeared next to every other treatment twice in both rows and columns, thus balancing out treatment interactions. The treatments were: (1) maize intercropped with *D. uncinatum* with or without 120 kg nitrogen/ha, (2) a maize monocrop with or without 120 kg nitrogen/ha, and (3) a maize monocrop with artificial ground shading made of maize straw (Hybrid 513) with or without 120 kg nitrogen/ha. The *S. hermonthica* seed level in each plot, before and after each cropping season, was measured at the National Sugar Research Centre, Kenya

Agricultural Research Institute, Kibos, Kenya, using the method described by Eplee and Norris (1990). Nitrogen content in each plot, before and after each cropping season, was measured at the National Agricultural Research Laboratory, Kenya Agricultural Research Institute, Nairobi, Kenya, using the Kjeldahl method (Shugar et al., 1981). During the experiment, light intensity, soil temperature, and humidity were monitored four times in all treatments, when the maize was 30, 60, 90, and 120 days old, and the results were averaged. Emergence and attachment of *S. hermonthica* to maize were measured, and maize plant height and cob yield were recorded.

Pot Culture Experiments. An experiment to investigate the effect of nitrogen availability in soil on witchweed infestation was conducted in a screenhouse by planting maize (Hybrid 513) and *D. uncinatum* together in pots (20 cm diam.) with autoclaved soil to which nitrogen (at the rate of 0, 30, 60, and 120 kg/ha) had been added. The tests were conducted with or without the presence of a nitrogen-fixing bacterium, *Rhizobium* sp., inoculum CB 627 (the taxonomy of this group is ambiguous and has not yet been assigned a species by molecular sequencing). The soil in each pot was infested with approximately 3000 *S. hermonthica* seeds, and emergence of witchweed in each pot and height of maize plants were measured at intervals.

To investigate the possibility of an allelochemical mechanism for *S. hermonthica* suppression, an aqueous solution of chemical components, eluting from 20 cm pots in which *D. uncinatum* plants were growing, was used to irrigate maize planted in soil infested with approximately 3000 *S. hermonthica* seeds/pot. To compare the effect of fixed nitrogen with that of the allelochemicals alone, *D. uncinatum* was planted with or without the nitrogen-fixing bacterium, *Rhizobium* sp. (CB 627). Autoclaved soil was used in all experiments, and no additional nitrogen was applied. The pots containing *D. uncinatum*, which received distilled water at a rate of 1.25 ml/min, were placed on shelves, thus allowing the flow of water by gravity through the pots into the maize pots situated below (Figure 1). Comparisons were made between maize plants irrigated by root eluates from *D. uncinatum* (with or without *Rhizobium* sp.) and those irrigated by water passing through pots containing only autoclaved soil (with or without *Rhizobium* sp.). Emergence and attachment of *S. hermonthica* to maize, and maize plant heights, were monitored in all treatments.

Laboratory Experiments. For surface sterilization and preconditioning of *S. hermonthica*, seeds were soaked in a 1% NaOCl solution (commercial bleach diluted fivefold) for 6–8 min with continuous shaking, rinsed with distilled water five times, and 30–40 seeds transferred to glass fiber disks (1.1 cm diam., GF/A). Each disk was wetted with distilled water (50 μl), and 10 such discs were placed in a plastic Petri dish (9 cm diam.), wrapped with Parafilm to prevent the disks from drying, placed in a polythene bag, and incubated at 25°C for 14 days.

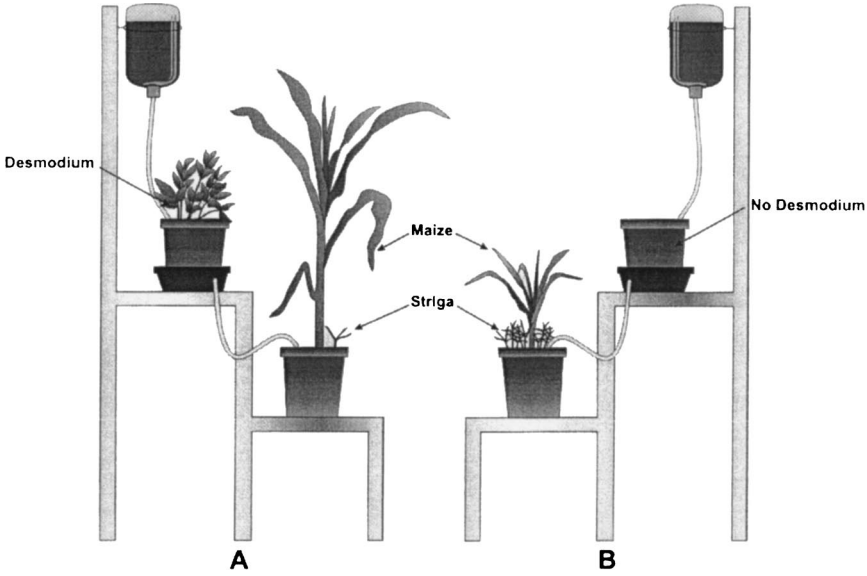


FIG. 1. Diagram of experiment to investigate the allelochemical mechanism of *Desmodium uncinatum* in suppressing *Striga hermonthica* infestation of maize. Comparison was made between maize plants irrigated by root eluates of *D. uncinatum* (A) with those irrigated by water passing through pots containing only autoclaved soil (B).

For the germination assay, glass fiber disks containing preconditioned *S. hermonthica* seeds (30–40/disk) were placed on filter paper (Whatman No. 1) to remove most of the moisture. The disks were returned to the Petri dish, and the test solution (50 μl) was applied to each disk. The Petri dish was wrapped with Parafilm and placed in an incubator at 35°C. Germinated seeds were counted under a dissecting microscope after 24–48 hr.

For the haustorium growth assay, haustorium lengths from the germinated *S. hermonthica* seeds were measured with an ocular meter (calibrated at $\times$20 magnification) mounted on a dissecting microscope 24 hr after the germination assay. The Petri dish containing germinated seeds was then placed in an incubator at 25°C and the haustorium length measured again after a further 24 hr.

For the collection of chemicals exuded from roots, *D. uncinatum* plants were grown in autoclaved soil (3 kg) containing different numbers of *S. hermonthica* seeds (0, 350, 700, and 1400/kg soil) in plastic pots (20 cm diam.) for varying periods (10, 15, 19, 20, 35, 45, 55, 69 days). At the end of each period, the soil was carefully washed off and the *D. uncinatum* plants (eight per treatment) transferred to glass columns (3.5 cm diam.) containing distilled water (20 ml) to collect the chemicals exuded by the cleaned roots. The aqueous exudate was assayed after

24 hr. Maize plants (four per treatment) were similarly grown in soil (3 kg) for two weeks, washed, and transferred to glass columns (3.5 cm diam.). These samples were used as the control.

For both field and pot experiments, differences in means were analyzed using one-way analysis of variance (ANOVA), and means were compared using Tukey's studentized range test (SAS Institute, 1996). Data in Table 3 (below) were analyzed using Student's *t*-test. In all studies, a $P \leq 0.05$ indicated significance.

RESULTS

In the field, intercropping of maize with the fodder legumes *D. uncinatum* and *D. intortum* significantly reduced *S. hermonthica* infestation, when compared to the maize monocrop, and significantly increased maize yields (Table 1). Of the other legumes tested, only cowpea gave a significant reduction in *S. hermonthica* infestation, but significantly less than the *Desmodium* spp. and with no increase in the maize yield.

The nitrogen and shading treatments, and the combined nitrogen and shading treatment, gave some suppression of *S. hermonthica* as compared to the maize monocrop plots (Table 2). However, a significantly greater reduction in *S. hermonthica* infestation was achieved by the *D. uncinatum* intercrop and the combined *D. uncinatum* and nitrogen treatments. As expected, there was a strong correlation between the numbers of witchweed plants per maize plant and the maize plant height and crop yields, and for both seasons, *D. uncinatum* had a greater effect on these than all the other treatments. Although shading had a significant effect on the *S. hermonthica* infestation, particularly in the 1999–2000 short rains, there was no commensurate increase in maize yield. A significant reduction in the numbers of *S. hermonthica* seeds in the soil was recorded in plots where *D. uncinatum* had been intercropped with maize (Table 3), with other treatments showing no change or a significant increase in the seed count. The only treatment showing a consistent

TABLE 1. EFFECTS OF VARIOUS LEGUME INTERCROPS ON *Striga hermonthica* INFESTATION AND MAIZE YIELDS IN FIELD PLOTS[a]

Treatment	*S. hermonthica*/63 maize plants	Maize yield (t/ha)
Maize monocrop	153.8 a	3.4 a
Maize and soybean	141.3 a	4.1 a
Maize and sun hemp	98.7 a	3.8 a
Maize and cowpea	67.6 b	3.6 a
Maize and *Desmodium uncinatum*	4.2 c	5.8 b
Maize and *D. intortum*	3.9 c	6.0 b

[a]Numbers in a column followed by a different letter are significantly different ($P < 0.5$) by Tukey's studentized range test. Data are means of six replications.

TABLE 2. EFFECTS OF NITROGEN, SHADING, AND *Desmodium uncinatum* INTERCROP ON *Striga hermonthica* INFESTATION, MAIZE PLANT HEIGHT, AND YIELDS IN FIELD PLOTS[a]

Treatment	*S. hermonthica*/ 63 maize plants	Maize plant height (m)	Maize yield (t/ha)
1999 Long rains			
Maize monocrop	459 a	1.61 a	3.62 a
Maize + shading	181 b	1.93 b	3.65 a
Maize + nitrogen (N)	245 ab	1.97 b	4.30 ab
Maize + shading + N	85 c	1.94 b	4.48 b
Maize + *D. uncinatum*	27 d	1.98 b	4.70 bc
Maize + *D. uncinatum* + N	8 d	2.19 c	5.04 c
1999–2000 Short rains			
Maize monocrop	769 a	1.67 a	2.53 a
Maize + shading	286 b	1.72 a	3.31 a
Maize + nitrogen (N)	486 ab	1.91 b	4.84 b
Maize + shading + N	113 c	1.94 b	5.00 b
Maize + *D. uncinatum*	13 d	2.09 c	5.20 bc
Maize + *D. uncinatum* + N	3 d	2.11 c	5.52 c

[a]Numbers in a column followed by a different letter are significantly different ($P < 0.5$) by Tukey's studentized range test. Data are means of six replications.

rise in the level of available nitrogen over both seasons was the maize/*Desmodium* intercrop (1999 short rains: +108.5%, $P < 0.01$; 1999–2000 long rains: +19.9%, $P < 0.05$). Data for soil temperature, light intensity and relative humidity in the plots are shown in Table 4.

TABLE 3. EFFECTS OF NITROGEN, SHADING, AND *Desmodium uncinatum* INTERCROP ON NUMBERS OF *Striga hermonthica* SEEDS IN SOIL (FIELD TRIALS, SUCCESSIVE SEASONS ON SAME PLOTS)[a]

	S. hermonthica seeds/250 g soil (*N*)					
	1999 short rains			1999–2000 long rains		
Treatment	Before	After	%change	Before	After	% change
Maize monocrop	253	421	+66.4**	421	607	+44.2**
Maize + shading	114	157	+37.7*	157	203	+29.3*
Maize + nitrogen (N)	239	256	+7.1 NS	256	298	+16.4*
Maize + shading + N	184	179	−2.7 NS	184	159	−11.2 NS
Maize + *D. uncinatum*	107	75	−29.9*	75	49	−34.7 NS
Maize + *D. uncinatum* +N	105	81	−22.8*	81	44	−45.7*

[a]Numbers before and after each season are significantly different at: $^{*}P < 0.05$, $^{**}P < 0.01$ by Student's *t*-test; NS = not significant. Data are means of six replications (each replication comprises 10 soil samples).

TABLE 4. EFFECTS OF SHADING AND *Desmodium uncinatum* INTERCROP ON SOIL TEMPERATURE (ST), RELATIVE HUMIDITY (RH), AND LIGHT INTENSITY (LI) IN MAIZE PLOTS DURING 1999 LONG RAINS[a]

Treatment	ST (°C)	RH (%)	LI (lux)
Maize monocrop	27.2 a	58.7 a	0.2 a
Maize + shading	26.8 ab	58.4 a	0.004 b
Maize + *D. uncinatum*	26.6 b	65.4 b	0.0 c

[a]Numbers in a column followed by a different letter are significantly different ($P < 0.5$) by Tukey's studentized range test. Data are means of six replications; each replication is a mean of four observations at different growth stages of maize.

The pot experiment to investigate the effects of nitrogen and *D. uncinatum* on suppression of *S. hermonthica* infestation in maize demonstrated that, in control pots, high levels of nitrogen application reduced and delayed emergence (Table 5). However, the effect of *D. uncinatum*, with or without nitrogen-fixing bacteria, was significantly greater in this regard compared to the nitrogen application alone. At all stages of maize development, the height of maize plants intercropped with *D. uncinatum* was greater than that of control plants with or without nitrogen (Table 6).

The dramatic effect of the aqueous solution of chemicals eluting from pots in which *D. uncinatum* plants were growing (Figure 1) on suppression of *S. hermonthica* infestation, and the concomitant increase in maize plant height, are shown in Figures 2 and 3. The aqueous samples of chemicals exuded by cleaned *D. uncinatum* roots were tested in laboratory experiments to investigate the mode of

TABLE 5. SUPPRESSION OF *Striga hermonthica* INFESTATION OF MAIZE PLANTS GROWN IN POTS, AT VARIOUS LEVELS OF NITROGEN, WITH OR WITHOUT *Desmodium uncinatum* AND NITROGEN-FIXING BACTERIA

	No. *S. hermonthica* plants/25 pots[a]											
	Maize + *D. uncinatum* + *Rhizobium* sp.				Maize + *D. uncinatum*				Maize (control)			
Nitrogen level (kg/ha)	0	30	60	120	0	30	60	120	0	30	60	120
Maize plant age (days)												
20	0	0	0	0	0	0	0	0	55	5	5	8
30	0	0	0	0	0	0	0	0	163	42	24	14
40	0	0	1	0	0	0	0	0	287	78	10	7
50	3	3	0	0	0	0	1	4	362	133	63	12
60	1	1	0	1	0	0	2	3	452	258	190	39
75	0	2	4	5	0	0	1	3	441	427	447	172

[a]3000 seeds per pot. Data are means of 50 replications.

TABLE 6. HEIGHT OF MAIZE PLANTS GROWN IN POTS IN *Striga hermonthica* INFESTED SOIL, AT VARIOUS LEVELS OF NITROGEN, WITH OR WITHOUT *Desmodium uncinatum* AND NITROGEN-FIXING BACTERIA[a]

Treatment	Maize plant height (cm)							
	35 days				60 days			
Nitrogen level (kg/ha)	0	30	60	120	0	30	60	120
Maize (control)	30 a	39 a	40 a	53 a	40 a	54 a	81 a	109 a
Maize + *D. uncinatum*	58 b	69 b	71 b	84 a	119 b	144 b	158 b	179 b
Maize + *D. uncinatum* + *Rhizobium* sp.	63 b	76 b	78 b	88 a	135 c	183 c	186 c	197 c

[a]Numbers in a vertical column followed by a different letter are significantly different ($P < 0.5$) by Tukey's studentized range test. Data are means of 50 replications.

action. There was no evidence for the production of a germination inhibitor by *D. uncinatum*, since the root exudate induced germination of *S. hermonthica* as effectively as the maize root exudate (Table 7). Indeed, it would appear that, in the initial stages (days 15–19), the *D. uncinatum* root exudates were more effective in this aspect than the maize root exudate. Furthermore, exudates from roots of *D. uncinatum* grown in soil at increasing densities of *S. hermonthica* infestation did not subsequently inhibit germination of *S. hermonthica* seeds (Table 8). However, when the seeds were exposed to root exudates from *D. uncinatum* and examined

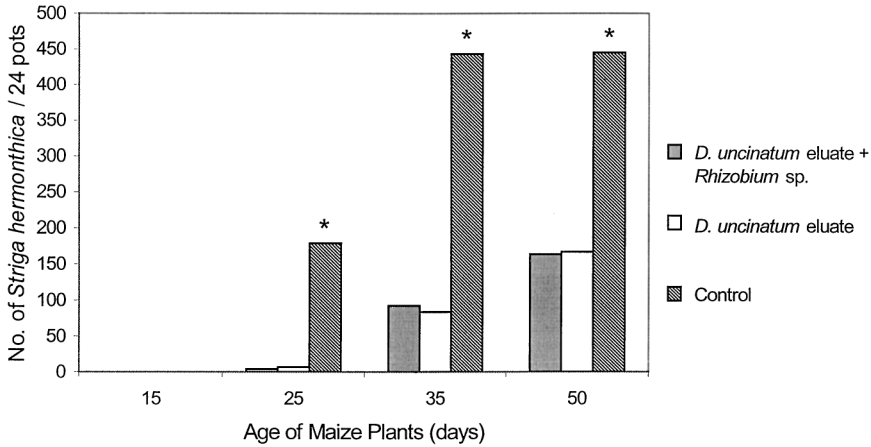


FIG. 2. Effect of *Desmodium uncinatum* root eluate (with and without nitrogen-fixing *Rhizobium* sp. bacteria) on numbers of *Striga hermonthica* parasitizing maize. Within each age group of maize, the treatment marked with an asterisk is significantly different ($P < 0.05$) by Tukey's studentized range test.

TABLE 7. GERMINATION OF *Striga hermonthica* SEEDS TREATED WITH AQUEOUS ROOT EXUDATES FROM *Desmodium uncinatum* OF DIFFERENT AGES

Treatment	Germination (%)				
Age of *D. uncinatum* (days)	15	19	35	55	69
Maize exudate + water (1:1)	21.7 ± 2.7 a	21.7 ± 2.7 a	47.9 ± 2.7 a	44.1 ± 2.6 a	44.1 ± 2.6 a
D. uncinatum exudate	50.5 ± 2.8 b	35.7 ± 3.4 b	50.8 ± 3.2 a	42.8 ± 3.6 a	54.7 ± 2.6 a
Maize exudate + *D. uncinatum* exudate (1:1)	51.7 ± 2.7 b	44.3 ± 3.9 b	50.3 ± 2.6 a	43.6 ± 3.0 a	44.4 ± 4.0 a
Distilled water	0.0 ± 0.0 c	0.0 ± 0.0 c	0.9 ± 0.5 b	0.0 ± 0.0 b	0.0 ± 0.0 b

[a]Numbers in a column followed by a different letter are significantly different ($P < 0.05$) by Tukey's studentized range test. Data are means of 10 replications, 30–40 seeds.

after 24 and 48 hr, the primary haustoria were significantly shorter relative to those exposed to maize exudates (Table 9). Inhibition of the primary haustoria was observed irrespective of whether the *D. uncinatum* had been grown in *S. hermonthica* infested or clean soil.

DISCUSSION

The initial field trial clearly demonstrated that intercropping with *D. uncinatum* and *D. intortum* produced significant and dramatic reductions in the infestation of maize plants by *S. hermonthica*. The overall yield increases result from a

TABLE 8. GERMINATION OF *Striga hermonthica* SEEDS TREATED WITH AQUEOUS ROOT EXUDATES FROM *Desmodium uncinatum* GROWN IN SOIL WITH VARYING *S. hermonthica* SEED DENSITIES[a]

S. hermonthica density (seeds/kg soil)	*D. uncinatum* germination (%)	
	10 days old	20 days old
1400	19.7 ± 3.2 a	40.5 ± 3.0 ab
700	26.0 ± 3.3 a	43.5 ± 2.0 ab
350	21.4 ± 3.1 a	32.1 ± 4.4 b
0	20.4 ± 2.5 a	47.0 ± 4.3 a
Maize exudate	27.8 ± 3.2 a	38.2 ± 3.4 ab
Distilled water	0.0 ± 0.0 b	1.0 ± 0.0 c

[a]Numbers in a column followed by a different letter are significantly different ($P < 0.05$) by Tukey's studentized range test. Data are means of 10 replications, 30–40 seeds.

TABLE 9. GERMINATION AND HAUSTORIAL DEVELOPMENT OF *Striga hermonthica* SEEDS TREATED WITH AQUEOUS ROOT EXUDATES FROM *Desmodium uncinatum* GROWN IN *S. hermonthica*-INFESTED OR CLEAN SOIL[a]

Treatment	Germination (%)[b]	Haustorial development			
		24 hr		48 hr	
		Haustorium length (mm)	Inhibition[c] (%)	Haustorium length (mm)	Inhibition[c] (%)
D. uncinatum exudate (+*S. hermonthica)*	39.3 ± 3.2 a	0.65 ± 0.04 a	18.8	0.85 ± 0.05 a	24.1
D. uncinatum exudate (−*S. hermonthica)*	43.0 ± 2.5 a	0.69 ± 0.03 a	13.8	0.75 ± 0.05 a	33.0
Maize exudate (control)	43.0 ± 2.5 a	0.80 ± 0.04 a	0.0	1.12 ± 0.06 b	0.0
Distilled water	0.0 ± 0.0 b				

[a] Numbers within a column followed by a different letter are significantly different ($P < 0.05$) by Tukey's studentized range test. Inhibition of haustorial development by *D. uncinatum* exudate is expressed relative to the mean length of haustoria treated with maize exudate.
[b] Data are means of 10 replications, 30–40 seeds.
[c] Data are means of 30 replications.

combination of witchweed and stem borer control, the latter arising from the use of the *D. uncinatum* intercrop to repel gravid stem borers from the maize. However, these data are presented as an indication of the ability of the overall strategy to address both aspects of the maize pest complex, with subsequent studies directed solely at control of *S. hermonthica*. Many claims have been made for the value of intercropping with other plants, for example, *Celosia argentina* L. (Amaranthaceae) and *Dolichos lablab* L. (Fabaceae), in witchweed control, but the effects reported are weak (e.g., Carson, 1986). Indeed, some suggested intercrops such as sweet potato, *Ipomoea batatus* L. (Convolvulaceae) (Oswald et al., 1999), would present considerable competition to the maize itself.

The use of *Desmodium* species to control parasitic witchweeds is showing promise. This has stimulated further investigations into the mode of action, for which a number of possible mechanisms have been considered, i.e., by increasing the available nitrogen in the soil, by offering shade, or through an allelopathic effect caused by chemicals released from *Desmodium* spp. roots. Such mechanisms are known to give some control of witchweeds (Press and Gurney, 2000). In the context of allelopathy for root-derived chemicals, three possible mechanisms were investigated: (1) inhibition of *S. hermonthica* seed germination by an exudate from *D. uncinatum*; (2) production by *D. uncinatum* of a germination inhibitor following attempts by *S. hermonthica* seedlings to attach to the plant; (3) inhibition of growth of the primary and/or upper haustoria.

Although, in the field, the nitrogen and shading treatments and the combination of nitrogen and shading suppressed *S. hermonthica* emergence, maximum suppression was provided by *D. uncinatum* and the combined *D. uncinatum* and nitrogen treatment. There is documented evidence of suppression of witchweed by added nitrogen sources (Mumera and Below, 1993; Press and Gurney, 2000). *D. uncinatum* is capable of fixing 100–180 kg nitrogen/ha under Kenyan conditions, and there is evidence that some of the nitrogen is passed to the companion plant (Muyekho *et al*., unpublished report). It has also been proposed that the suppression of witchweed by a leafy intercrop may be due to shading effects (Parker and Riches, 1993; Press and Gurney, 2000). This may reduce temperature and raise humidity over the emerging witchweed plants, thus reducing transpiration and the supply of nutrition from the host plant. Although, in this study, there were benefits from nitrogen and shading on suppression of *S. hermonthica* and growth of maize in the field, the laboratory pot experiments clearly demonstrated an additional allelopathic effect associated with *D. uncinatum*. The greater effect of *D. uncinatum* compared to physical and nutritional factors indicated an additional activity strongly suggestive of allelopathy, which is also reflected in the depletion of viable *S. hermonthica* seeds in the soil after both cropping seasons.

An allelopathic mechanism was demonstrated by the pot experiments described in Figures 1–3, i.e., water-soluble chemical components associated with *D. uncinatum* roots are responsible for suppression of *S. hermonthica* infestation. The role of chemicals specifically released by cleaned *D. uncinatum* roots was further investigated, but there was no evidence of germination inhibition, which had

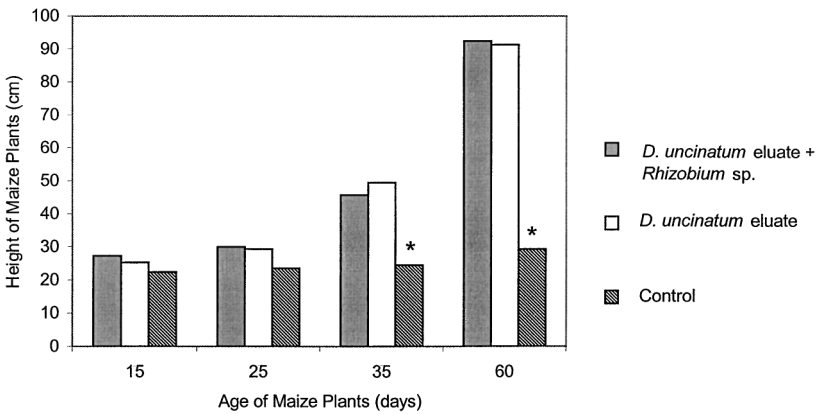


FIG. 3. Effect of *Desmodium uncinatum* root eluate (with and without nitrogen-fixing *Rhizobium* sp. bacteria) on height of maize plants growing in *Striga hermonthica* infested soil. Within each age group of maize, the treatment marked with an asterisk is significantly different ($P < 0.05$) by Tukey's studentized range test.

originally been expected. The first possible mechanism (1, above) is, thus, discounted. Indeed, there is evidence for the production of a potent germination stimulant by root exudates of *D. uncinatum*. The second possibility (2, above) is discounted because of the demonstration that root exudates from *D. uncinatum* growing in soil infested with *S. hermonthica* at increasing densities did not subsequently inhibit germination of *S. hermonthica* seeds. The fact that chemical components of *D. uncinatum* root exudates gave a significant inhibition of haustorial growth after 48 hr demonstrated a mechanism (3, above), in which haustorial development in the parasitic process is implicated.

The discovery of the potential of *Desmodium* spp. in the suppression of *S. hermonthica* infestation has opened the possibility for new practical witchweed control methods commensurate with East African subsistence farming (Khan et al., 2000). The allelopathic effect of chemicals exuded from the roots that interfere with haustorial development, combined with the potent chemical stimulants causing suicidal germination, provide not only direct witchweed control, but also a significant depletion of viable seeds in the soil. Full chemical elucidation of the allelopathic agents is ongoing, but fractionation by HPLC has already demonstrated that the germination stimulants in *D. uncinatum* root exudates can be isolated from the haustorial inhibitors. Certain novel polyphenolic compounds have been tentatively identified from active fractions and await confirmation of activity and structures in synthesis studies.

Research into the factors stimulating germination of flowering parasitic weeds has been under way for a number of years (e.g., Netzly et al., 1988), and work on eliciting suicidal germination is ongoing (e.g., Wigchert et al., 1999). In addition, a great deal is known about the chemistry involved (Sugimoto et al., 1998; Welzel et al., 1999). However, no practical exploitation in weed control has yet ensued. The sophisticated mode of action demonstrated here, comprising both germination stimulation and haustorial inhibition, when fully elucidated, may give exploitable leads that are urgently needed, not only in subsistence agriculture, but also to address future world demands in agricultural production and in developing new approaches for molecular biology in weed control (Gressel, 2000).

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ODORANT-BINDING PROTEINS FROM A PRIMITIVE TERMITE

YUKO ISHIDA,[1] VICKY P. CHIANG,[1] MICHAEL I. HAVERTY,[2] and WALTER S. LEAL[1,*]

[1]*Honorary Maeda-Duffey Laboratory, Department of Entomology*
University of California,
Davis, California 95616

[2]*Chemical Ecology of Forest Insects, Pacific Southwest Research Station*
Forest Service, USDA, P.O. Box 245
Berkeley, California 94701

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Abstract—Hitherto, odorant-binding proteins (OBPs) have been identified from insects belonging to more highly evolved insect orders (Lepidoptera, Coleoptera, Diptera, Hymenoptera, and Hemiptera), whereas only chemosensory proteins have been identified from more primitive species, such as orthopteran and phasmid species. Here, we report for the first time the isolation and cloning of odorant-binding proteins from a primitive termite species, the dampwood termite, *Zootermopsis nevadensis nevadensis* (Isoptera: Termopsidae). A major antennae-specific protein was detected by native PAGE along with four other minor proteins, which were also absent in the extract from control tissues (hindlegs). Multiple cDNA cloning led to the full characterization of the major antennae-specific protein (ZnevOBP1) and to the identification of two other antennae-specific cDNAs, encoding putative odorant-binding proteins (ZnevOBP2 and ZnevOBP3). N-terminal amino acid sequencing of the minor antennal bands and cDNA cloning showed that olfaction in *Z. n. nevadensis* may involve multiple odorant-binding proteins. Database searches suggest that the OBPs from this primitive termite are homologues of the pheromone-binding proteins from scarab beetles and antennal-binding proteins from moths.

Key Words—Odorant-binding protein, chemosensory protein, *Zootermopsis nevadensis nevadensis*, Isoptera, Termopsidae, olfaction, dampwood termite.

* To whom correspondence should be addressed. E-mail: wsleal@ucdavic.edu

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INTRODUCTION

Insects perceive the world through small molecules that carry essential information about nestmates, food sources, intruders, and many other environmental cues. These hydrophobic semiochemicals are solubilized by odorant-binding proteins (OBPs) and transported through an aqueous environment (sensillar lymph) to the olfactory receptors, where the signal transduction starts (Kaissling, 2001). OBPs have been identified and/or cloned from various insect orders, such as Lepidoptera, Coleoptera, Diptera, Hymenoptera, and Hemiptera (Vogt et al., 2002). These proteins, subdivided into pheromone-binding proteins (PBPs) and general odorant-binding proteins (GOBPs), all have a hallmark of six conserved cysteine residues forming three disulfide bridges (Leal et al., 1999; Scaloni et al., 1999), which are essential for the rigidity of their three-dimensional structures (Horst et al., 2001; Sandler et al., 2000). A third class of small proteins has been identified in several sensorial organs from a number of insect orders (Lartigue et al., 2002). Although they may function as carrier proteins (Lartigue et al., 2002 and references therein), they have been separated into a group of chemosensory proteins (CSP), characterized by four cysteine residues and with low sequence similarity to OBPs. Interestingly, a thorough search for olfactory proteins in insect orders other than Lepidoptera (Marchese et al., 2000 and references therein) revealed that species from primitive insect orders, such as Orthoptera and Phasmatodea, possess CSPs, but not OBPs. This study was aimed at the identification of olfactory proteins from the primitive Dictyoptera, which includes the orders Mantodea (mantids), Blattodea (cockroaches) and Isoptera (termites). Here, we report the identification and cloning of OBP homologues in the dampwood termite, *Zootermopsis nevadensis nevadensis* HAGEN (Isoptera: Termopsidae).

METHODS AND MATERIALS

Protein Analyses. Termite colonies were collected from ponderosa pine (*Pinus ponderosa* DOUGL. EX LAWS) stumps on the Eldorado National Forest near Placerville, California. Colonies were maintained in culture in 41 plastic containers (Rubbermaid Inc., Wooster, Ohio) for 5–6 months. Workers were anesthetized on ice and their antennae and hindlegs were collected with forceps under a microscope. After homogenizing in 10 mM Tris-HCl, pH 8 with an ice-cold Dounce tissue grinder (Wheaton), homogenates were centrifuged twice at 13,500 $\times$ g for 10 min at 4°C. The supernatants were concentrated by centrifugation under vacuum, analyzed by 15% native polyacrylamide gel electrophoresis (15% PAGE); bands were visualized with Coomassie blue R-250. For N-terminal amino acid microsequencing, proteins were separated by PAGE, transferred to polyvinyl difluoride (PVDF) membranes using electroblotting, identified bands were cut out and analyzed on a Procise Protein Sequencing System (Applied Biosystems).

cDNA Cloning and RT-PCR. Total RNA was extracted by TRIzol (Invitrogen) including 200 ng of glycogen (Invitrogen) from 130 antennae of workers. First strand cDNA was synthesized by using SuperScript II (Invitrogen) as transcriptase and SMART RACE cDNA Amplification Kit (Clontech). Degenerate primer,

5′-GC(A/C/G/T)AA(A/G)GA(A/G)GT(A/C/G/T)GA(C/T)GA(A/G)AA-3′

was designed on the basis of amino acid sequence of N-terminal region (AKEVDEK) of isolated antennae-specific protein. GCGTTTTTTTTTTTTTT (GCGT15) primer was used as a reverse primer. *Taq* DNA Polymerase in Storage Buffer B (Promega) was used as *Taq* polymerase. PCR were performed as follow: 70 cycles of 94°C for 1 min, 40°C for 2 min and 72°C for 3 min. PCR product was ligated into pBluescript SK (+) (Stratagene) and sequenced at an automated DNA sequencing facility (Davis Sequencing). More than 4 independent clones were sequenced to eliminate possible PCR mutations. For 5′-RACE a gene specific primer (Z1-r),

5′-CGATGGCCCTCTTCCACGATCTCTGGCGGT-3′,

was designed, and PCR was carried out as in the instruction manual of SMART RACE cDNA Amplification Kit. For cloning two other cDNAs, we used a N-terminal degenerate primer, GCGT15 primer and 4 gene-specific primers,

5′-CTCAACATGACTGGCAGTTTCCCCGATGAG-3′ (Z2-f),

5′-CCGAACGCCTCCTTCAACTTAGCGGCGACC-3′ (Z2-r),

5′-AATGAAGTGCTCGATCACCGGGACAACAGC-3′ (Z2-r2)

and

5′-GGAAACCCTTGCAGCTTCTCCACCACTGTC-3′ (Z3-r)

and performed PCR-cloning described above. Molecular mass, isoelectric point, BLAST database search, and ClustalW alignment of amino acid sequences were performed by MacVector (Accelrys). Signal peptide was predicted by SignalP V1.1 (http://www.cbs.dtu.dk/services/SignalP/). For RT-PCR, cDNAs were synthesized from total RNA extracted from antennae and legs as described above. The following combinations of gene-specific primers were used to detect the transcript of the cloned odorant-binding proteins:

Z1-f, 5′-GTGTCATGGTCGAACTCATGGCTCTCAACG-3′

and Z1-r (for ZnevOBP1), Z2-f and Z2-r (for ZnevOBP2), and

Z3-f, 5′-ACTTCGGCAGTTTCCCGGATGAGTCCGACC-3′,

and Z3-r (for ZnevOBP3). For detection of actin transcript (control), two degenerate primers were used: Actin-1,

5′;-AA(C/T)TGGGA(C/T)GA(C/T)ATGGA(A/G)AA-3′

and Actin-2,

5′-GCCAT(C/T)TC(C/T)TG(C/T)TC(A/G)AA(A/G)TC-3′.

To amplify OBP cDNAs 25 cycles of a stepwise program (94°C for 5 sec; 55°C for 10 sec; 72°C for 3 min) were carried out. Actin cDNA was amplified by 45 cycles of a stepwise program (94°C for 1 min; 45°C for 2 min; 72°C for 3 min). PCR products were separated by 1% agarose gel.

RESULTS AND DISCUSSION

Our protein-based approach leads us to the identification of olfactory proteins by comparing the profile of proteins isolated from antennae and control tissues. A major antennae-specific protein was identified by native PAGE analyses of proteins from workers of *Z. n. nevadensis* (Fig. 1A).

The putative olfactory protein migrated faster than our reference protein, the pheromone-binding protein from *Bombyx mori*, BmPBP, and showed an N-terminal amino acid sequence (LTGRAFERAKEVDEK-RS; -indicates no signal) with 55% sequence identity (70% similarity) to the previously identified olfactory proteins (Pam6m and Pam6f) from the American cockroach, *Periplaneta americana* (Picimbon and Leal, 1999). In order to obtain further sequence information, the cDNA encoding this protein was cloned. A single-banded cDNA (550 bp) was amplified with a degenerate primer (based on the N-terminal amino acid sequence) and an GCGT15 primer. With this partial sequence available, we designed a gene specific primer, determined the 5′-region, and obtained the full length of cDNA, which encoded a protein with 151 amino acid residues (Fig. 1B). The predicted cleavage site of the signal peptide was in agreement with the N-terminal amino acid sequence of the isolated protein. Calculated molecular mass and the isoelectric point for the mature protein were 14,849 Da and pI 4.84, respectively. BLAST database search indicated considerable sequence similarity with previously identified odorant-binding proteins, with the highest scores (30–33% identity) to odorant-binding proteins from the scarab beetles *Exomala orientalis*, *Anomala osakana*, *Popillia japonica*, *Anomala cuprea*, and *A. octiescostata*, *Phyllopertha diversa*, *Heptophylla picea* and *Holotrichia parallela* (Deyu and Leal, 2002 and references therein). The sequence of a cockroach OBP, *Leucophaea maderae*, in the database (accession number AY116618) showed only 12% identity to ZnevOBP1. In addition to significant sequence similarity, the antennae-specific protein from *Z. n. nevadensis* possesses the hallmark of odorant-binding protein, six cysteine residues (at positions 16, 46, 50, 88, 97, and 106) (Fig. 1B). Hence,

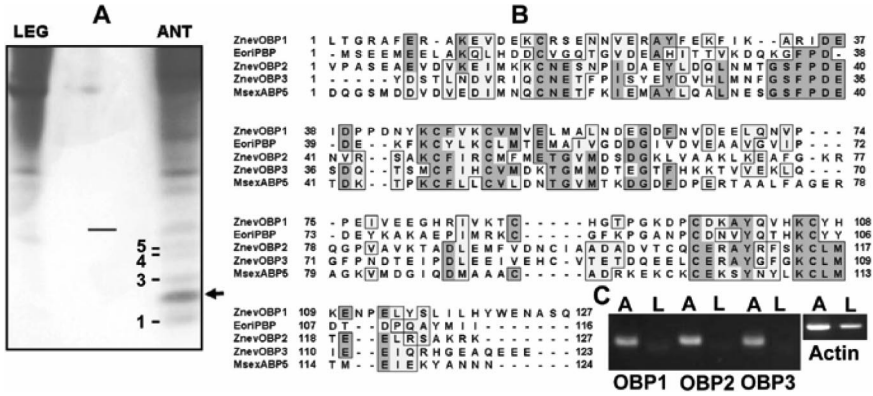


FIG. 1. Gel electrophoresis analysis of proteins extracted from *Z. n. nevadensis*, sequence alignment of deduced amino acids from the cloned OBP cDNAs, and RT-PCR analysis of transcription. (A) Soluble proteins analyzed by a 15% native PAGE and stained with Coomassie blue. LEG, control tissue; 100 midlegs-equivalent. ANT, 400 antennae-equivalent. Arrow indicates the major antennae-specific binding protein. Numbers are given to other minor antennae-specific proteins by their order of mobility. Migration of a reference protein, the pheromone-binding protein from *B. mori* (BmPBP), is denoted by a bar in the empty lane. A band with mobility similar to BmPBP was not antennae-specific. (B) Alignment of ZnevOBP1 with the protein in the database with the highest sequence identity, namely, the pheromone-binding protein from the Oriental beetle, *E. orientalis* (EoriPBP; AB040144). Alignment of ZnevOBP2 and ZnevOBP3 with the best fitting protein, i.e., the antennal-binding protein of *M.* sexta (MsexABP5; AF393498). For clarity, the putative signal peptide and the first 15 amino acid residues of MsexABP5 were omitted. The cDNA sequences for ZnevOBP1, ZnevOBP2, and ZnevOBP3 have been submitted to the GenBank database with accession numbers AY135381, AY135382, and AY135383, respectively. (C) RT-PCR data. cDNA fragments (ca. 100 bp) were amplified with gene-specific primers and antennal template (A), but not with template derived from legs (L). cDNA fragments (450 bp) of actin (control) were amplified using templates from both legs (L) and antennae (A).

we suggest that the isolated protein is an odorant-binding protein and named it ZnevOBP1.

While sequencing multiple clones for ZnevOBP1, we were able to identify two other cDNAs encoding putative odorant-binding proteins, which we named ZnevOBP2 and ZnevOBP3. One cDNA (ZnevOBP2) encoded a protein with 148 amino acid residues. The mature protein (127 amino acid residues) gave a calculated molecular mass and isoelectric point of 14,173 Da and pI 4.95, respectively. Interestingly, as the pheromone-binding protein from *Manduca sexta* (MsexPBP1) (Vogt et al., 2002), this protein has seven cysteine residues (Cys-17, 47, 51, 95, 104, 106, and 115) (Fig. 1B). The other cDNA (ZnevOBP3) encoded a

protein with 143 amino acid residues, the first 20 residue being the putative signal peptide. The calculated molecular mass and isoelectric point for the mature protein were 14,272 Da and pI 4.07, respectively. This protein possesses 6 cysteine residues in a pattern similar to that of ZnevOBP1, i.e., Cys-12, 42, 46, 88, 98, and 107. BLAST search indicated that ZnevOBP2 and ZnevOBP3 shared significant sequence similarity with several insect odorant-binding proteins, the highest scores being the antennal binding protein cloned from *M*. sexta MsexABP5 (AF393498), which showed 41 and 35% identity to ZnevOBP2 and ZnevOBP3, respectively (Fig. 1B).

In order to test whether the cloned cDNAs were related to the minor antennae-specific proteins from *Z. n. nevadensis* (see Fig. 1A), we attempted to determine the N-terminal amino acid sequences of bands 1, 3, 4, and 5. Given the scanty amounts of these proteins in the antennae, the obtained sequences were short, and the readings of the first amino acid residues were not reliable. However, the partial sequences showed that these minor antennae-specific bands are not encoded by the cloned cDNAs. Bands 1 and 3 gave partial sequences Ser-Pro-Leu/Phe-Asp and Gln-Met-Glu-Glu starting from the second and third residues, respectively. On the other hand, bands 4 and 5 gave sequences Val-Lys-Gln and Met-Leu-Asn, starting from the fourth residue. None of these residues is identical to those at the same positions in ZnevOBP2 and ZnevOBP3.

Two lines of evidence suggest that termites have multiple olfactory proteins. First, the minor bands 1, 3, 4, and 5 are specific to the antennae and show mobility similar to most acidic odorant-binding proteins identified to date; they are probably OBPs. Secondly, RT-PCR showed that ZnevOBP2 and ZnevOBP3 are also specific to antennae (Fig. 1C). In conclusion, the evidence from *Z. n. nevadensis* suggests that olfaction in termites (Isoptera) is mediated by multiple odorant-binding proteins.

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INTRODUCTION—SPECIAL ISSUE ON AQUATIC CHEMICAL ECOLOGY

Periodically the journal devotes an entire issue to a particular sub-discipline of chemical ecology. The last such volume was September 2000 [26(9)] when the topic was allelopathy. This current volume developed from a conversation between Mark Hay and John Romeo during the poster session at the International Society of Chemical Ecology annual meeting at Lake Tahoe in July 2001. Mini-reviews were solicited and a call for papers was advertised in the journal. What results here is a series of reviews that cover many important recent developments in the field and a group of papers that illustrate some of the exciting work being done in laboratories worldwide.

The mini-reviews and the direction and likely force of the next wave in aquatic chemical ecology are summarized in the Preface by Mark Hay. The contributed papers (seven selected from several that were submitted) range from studies of pheromones to induction of chemical defenses and to chemically mediated tritrophic interactions. The paper by Ichinose illustrates the importance of coupling biological and chemical studies. His work shows that age and body size of freshwater snails determine their alarm responses to predators, and this is apparently linked to a developmental production of chemicals. The role of bacterial metabolites is nicely shown in the work of Harder et al., who implicate their role in cueing settlement of larval polychaetes. Walenciak et al. demonstrate the potential biotic complexity of aquatic trophic interactions by showing that gut symbionts in aquatic moths are affected by the tannin-containing macrophytes included in their diets. Induced responses in aquatic organisms, as in terrestrial ones, take many forms. While Küpper et al. show that kelps challenged by a pathogen induce increased resistance via production of oligosaccharides, Thorton and Kerr indicate that unusual pseudopterosins (diterpene glycosides) are induced in gorgonian corals by decreasing UV radiation and heavy mollusk, but not fish, predation. Jung et al. show that a major secondary metabolite (the sesquiterpene caulerpenyne in this case) from a green alga found in reef habitats, undergoes metabolic transformation after wounding of the alga. Finally, Ingvarsdóttir et al. have shown that even lowly parasitic sea lice use pheromones to find mates!

A cursory look at the techniques and methods used by these workers shows not only the unique difficulties encountered in working with organisms from aquatic systems, but also how far the discipline's technology has evolved. The range

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includes traditional extraction and spectroscopic identification, freeze shocking and enzyme kinetics (Jung et al.), and PCR and sequence analysis for establishing genetic affinity (Walenciak et al.). The range of compounds encountered is varied even within this small sample of papers and includes most classes of natural products (phenolics, terpenoids, alkanes/alkenes/ketones, oligosaccharides). Only alkaloids are not represented. In many cases, the chemistry is unknown (Ichinose) or known in only a nonspecific general way (Ingvarsdóttir et al., Küpper et al., Walenciak et al.), but in others, the compounds (Thorton and Kerr, Harder et al.), and their metabolism (Jung et al.) are understood in significant detail.

The editors thank all those who contributed to this endeavor with special acknowledgment to the driving force of Mark Hay. We hope the issue will stimulate thinking and work in this exciting area.

John T. Romeo and Nancy M. Targett

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PREFACE

THE NEXT WAVE IN AQUATIC CHEMICAL ECOLOGY

MARK E. HAY*

School of Biology
Georgia Institute of Technology
Atlanta, Ga 30332-0230 USA

Aquatic chemical ecology is a recent, but dynamic and rapidly developing, discipline that has become a recognized field only within the last two decades. As an example, a 2001 book on marine chemical ecology (McClintock and Baker, 2001) lists thousands of references with nearly 90% being post-1982 and about 50% being post-1992. Prior to the 1980s, some chemists were exploring marine organisms for novel secondary metabolites and some ecologists were documenting patterns that appeared to be chemically-mediated, but it was uncommon for the chemistry and ecology to be rigorously merged. In the 1980s, marine chemists and ecologists began collaborating to explore types of chemically mediated interactions that had been investigated previously in terrestrial systems (e.g., consumer–prey interactions). These studies benefited greatly from the intellectual infrastructure of terrestrial investigations and models. However, aquatic investigations also provided increased depth to the general fields of chemical ecology, community ecology, and evolution by: (1) testing theories derived for terrestrial systems in evolutionary independent aquatic systems; (2) assessing the ecological function of secondary metabolites under field conditions where investigators did not need to rely on assumptions about the important consumers, primary selective pressures, or relative roles of consumers vs other selective agents; and (3) integrating the specific metabolites and interactions being investigated into a larger community-based framework so the cascading effects of chemically mediated interactions on marine diversity and community organization could be understood. Most initial studies focused on understanding the ecological or evolutionary consequences of prey chemical defenses—an area well represented by terrestrial investigations. This

* E-mail: mark.hay@biology.gatech.edu

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provided opportunities for a host of marine–terrestrial contrasts that advanced our understanding of chemical ecology in both aquatic and terrestrial systems.

In addition to studies of defense, investigations of chemically mediated foraging, habitat selection, induction of metamorphosis, host–symbiont interactions, and mate tracking and recognition have been conducted in aquatic systems. To be ecologically realistic, these investigations often demand complex integration of behavior, sensory ecology, chemistry, hydrodynamics, and micro- or molecular biology. Achieving such integration is an obvious challenge, but significant advancements are being made, and the field seems poised to merge these disciplines in ways that will provide rigorous mechanistic insights into major processes regulating populations and affecting the structure and function of entire communities.

The mini-reviews presented in this issue attempt to demonstrate both the exceptional opportunities and significant challenges involved in aquatic chemical ecology. In recent years, comprehensive reviews focused on chemical-mediation of biotic interactions in aquatic systems have become a growth industry (e.g., Hay and Fenical, 1988; Davis et al., 1989; Paul, 1992; Pawlik, 1992, 1993; Hay, 1996; Weissburg, 2000; Zimmer and Butman, 2000; McClintock and Baker, 2001). Given these recent summaries, the mini-reviews requested for this issue were targeted at research areas in the early stages of development, but demonstrating great promise. Authors were challenged to be strategic rather than comprehensive in their coverage and to focus more on opportunities, challenges, and needs than on summarizing previous investigations. The mini-reviews, therefore, represent a terse synopsis of dynamic areas within the field, along with a call for focused investigations that will shape the future.

The reviews champion greater focus on: (1) the underlying physiological and molecular basis of metabolite synthesis, turnover, and deployment; (2) chemical mediation of interactions among microbes and between microbes and hosts; (3) understanding how hosts can use chemical signals to regulate microbial behavior (rather than survival via antibiotic activity) as a defense against enemies or to facilitate mutualist microbes; (4) an appreciation of the ontogeny of chemical defense among organisms with complex life-cycles, and the repercussions of this for the evolution of life history and behavioral traits; (5) understanding how the flow environment in which interactions occur may facilitate or hinder consumer foraging and prey escape via affects on transmission and detection of chemical signals; (6) employing a nonreductionist approach that tests for emergent responses to suites of multiple, and sometimes conflicting, chemical cues; and (7) elucidating the cascading effects of secondary metabolites on nontarget organisms and how these effects may impact larger scale patterns such as food-web structure, community composition, rates and pathways of biogeochemical cycles, and the coupling of aquatic with terrestrial systems.

Although the questions and approaches outlined in these mini-reviews are general, they are addressed in systems (e.g., freshwater, the plankton), life stages

(e.g., larvae), or organisms (e.g., marine microbes) where previous investigations have been adequate to demonstrate exciting possibilities, but where much progress is needed and now possible. The authors of these mini-reviews focus on exciting subdisciplines that are just beginning to flourish. It is hoped that the excitement and significant opportunities presented in these reviews will stimulate other investigators to accept the intellectual and methodological challenges noted by these authors and push the field forward to new frontiers.

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MINI REVIEW

CUED IN: ADVANCES AND OPPORTUNITIES IN FRESHWATER CHEMICAL ECOLOGY

ROMI L. BURKS*,[1] and DAVID M. LODGE[2]

[1]*Rhodes College, Biology Department*
2000 North Parkway
Memphis, Tennessee 38112, USA

[2]*University of Notre Dame*
Department of Biological Sciences
Notre Dame, Indiana 46556, USA

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Abstract—We focus this mini-review on how naturally occurring chemical cues mediate ecological interactions, especially interspecific competition and predation in freshwater communities. Although freshwater chemical ecology lags behind terrestrial and marine chemical ecology, we identify recent progress toward: (1) identifying the chemical composition of cues important in food web interactions, e.g., specific glucosinolates, benzyl succinoates, and lignoids as deterrents to herbivory on freshwater macrophytes; (2) employing a nonreductionist approach that tests for emergent responses to suites of multiple chemical cues, e.g., trade-offs in snail refuge-seeking behavior in the presence of chemical cues from both fish and crayfish; (3) investigating how abiotic forces, such as hydrodynamics, impact chemical communication across a broad spatial and temporal scale, e.g., drift responses of mayfly nymphs to whole-stream additions of trout cue; and (4) quantifying the importance of genetic variability, e.g., how chemical cues change selective pressures of local environments. The questions of interest in freshwater chemical ecology cross taxonomic boundaries; traverse broad spatial and temporal scales; demonstrate nonlinear, unpredictable results; and necessitate a multidisciplinary approach for adequate understanding.

Key Words—chemical cue, kairomone, identification, scale, communication, behavior, predator–prey, flow, genetic variability.

* To whom correspondence should be addressed. E-mail: burksr@rhodes.edu

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INTRODUCTION

Chemical cues are important signals in aquatic environments, where visual and auditory senses are often ineffective. As we illustrate in this review, chemical communication among freshwater species mediates many aspects of both predation and interspecific competition, forces that play key roles in determining community structure and ecosystem function. Recent advances in aquatic chemical ecology are enhancing our understanding of the connections between individual behavior and ecosystem function. We synthesize results at individual, population, and community levels, emphasizing the importance of investigations at multiple spatial and temporal scales.

Because terminology in the field of chemical communication is often ambiguous (Oldham and Boland, 1996), we explain our use of terms. We use the term "chemical cue" as a synonym for "allelochemical," a cue carrying information that mediates interactions between two organisms (*sensu* Dicke and Sabelis, 1988). Consistent with previous authors, we use the terms "kairomone" to indicate communication between different species and "pheromone" to indicate information relayed within a species (Wisenden and Millard, 2001). In addition, we discuss organisms in terms of their role as signalers versus receivers of infochemicals (*sensu* Dicke and Sabelis, 1988). Throughout the review, we address ecological interactions that are mediated by naturally occurring chemicals (not human-made chemicals). We also try to point out where the exact chemical structure of a cue is known versus generalized knowledge about groups of chemical compounds.

Several recent freshwater-oriented reviews overlap or focus on subsets of our topic: chemical communication (Brönmark and Hansson, 2000); alarm signaling (Chivers and Smith, 1998); predator–prey interactions (Kats and Dill, 1998); benthic interactions (Dodson et al., 1994); and planktonic interactions (Larsson and Dodson, 1993; Tollrian and Dodson, 1999). Rather than attempting to be comprehensive, we rely on these reviews, and emphasize selected publications of the past 5–10 years.

We first present patterns of recent publications that reveal what questions, organisms, and habitats have been studied. Second, we discuss specific advances that involve: (1) quantitative isolation and identification of chemical cues; (2) behavioral trade-offs that occur for organisms responding to multiple chemical cues; (3) abiotic influences that span spatial and temporal scales; and (4) characterization of underlying genetic variability of signalers and responders. Finally, we highlight remaining opportunities and challenges for research in this dynamic field.

PATTERNS OF RECENT PUBLICATION

With a literature survey using *Current Contents* and *Biological Abstracts* (Winspirs 4.0) from 1992 to 2001, we quantified the temporal trend of publications

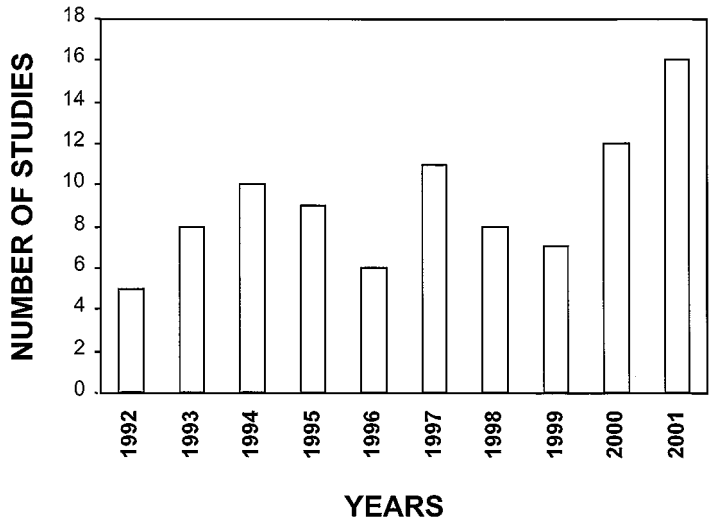


FIG. 1. Number of studies in freshwater chemical ecology from 1992 to 2001 that met our search criteria (in *Biological Abstracts* and *Current Contents*) with keywords: chemical cue, chemical communication, or kairomone.

relating to chemical communication in freshwater habitats. We classified the organisms in these publications as either senders or receivers (Dicke and Sabelis, 1988). Three keyword searches (chemical cue, chemical communication, kairomone) provided limitations for our survey. We limited our search using keywords that we believe freshwater ecologists would most likely use to explore the world of chemical ecology. This delineation limited our search to cues influencing relevant ecological interactions versus identification of nonrelevant or anthropogenic chemicals.

Our restricted search suggests that publications on freshwater chemical ecology are increasing at a slow rate (Figure 1). In addition, striking patterns emerge regarding the types of organisms that act as senders versus receivers of chemical cues (Figure 2). Although our literature search included broad classes of chemical cues, our results suggest that kairomones mediate the majority of species interactions in freshwater systems. Fish and predacious insects act largely as senders, while zooplankton comprise the most studied receivers. Available evidence suggests that some organisms, such as tadpoles, act only as receivers of cues from predators. While other organisms, such as predacious insects, may be both receivers of cues from larger predators as well as senders of their own cues to a lower trophic level, such as zooplankton (especially *Daphnia*).

Our survey confirms Kats and Dill's (1998) assertion that the majority of studies on aquatic chemical communication involve fish–zooplankton interactions. Furthermore, we may only identify some organisms as receivers, when, in

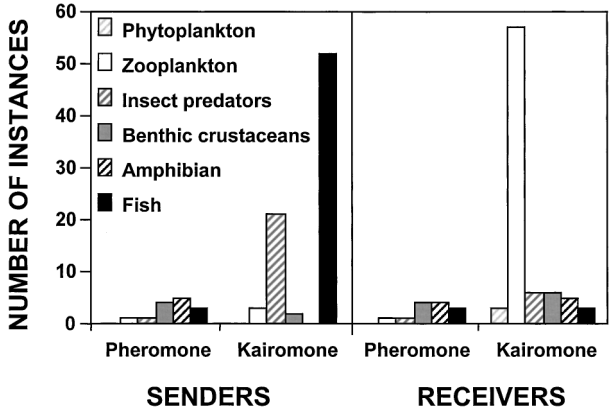


FIG. 2. Number of instances that different taxonomic groups appear as senders or receivers of chemical cues in studies identified from our literature search (1992–2001). Insect predators include both pelagic (Diptera: *Chaoborus*) and benthic (Odonata: *Enallagma*) organisms. Benthic crustaceans include amphipods, isopods, and crayfish. In most instances, fish act as senders and zooplankton serve as receivers.

actuality, those organisms are influencing other inconspicuous organisms in less well-studied interactions. On top of these documented complex patterns, considerable phenotypic plasticity and underlying genetic variability undoubtedly exists in how organisms respond to cues. These complexities indicate that freshwater chemical ecology will develop most quickly with an interdisciplinary approach (Oldham and Boland, 1996).

The patterns illustrated (Figures 1 and 2) represent both standing and flowing freshwater habitats. The duration and orientation of chemical cues may, however, depend partly on the type of freshwater habitat and the organisms present (Figure 3). In lotic systems (i.e., streams), chemical cues from mobile foragers such as trout (McIntosh et al., 1999) or crayfish (Moore and Grills, 1999) may be strongly unidirectional, diluting as they proceed downstream (although see Dahl et al., 1998). In contrast, recycling or renewal of chemical cues by less mobile organisms is more likely in lentic systems (i.e., macrophytes in lakes) (Figure 3). In addition, organisms showing strong site fidelity may also create areas in which cues are recycled. Finally, we might predict chemical cues to be relatively more important in aquatic systems that lack light, such as groundwater or cave environments. However, examples of chemical communication in subsurface organisms are, to our knowledge, nonexistent. We highlight below four promising avenues of recent advances that point the way toward enhanced interdisciplinary research. Future interdisciplinary endeavors will illustrate the importance of chemical ecology in mediating population, community, and ecosystem processes in freshwaters.

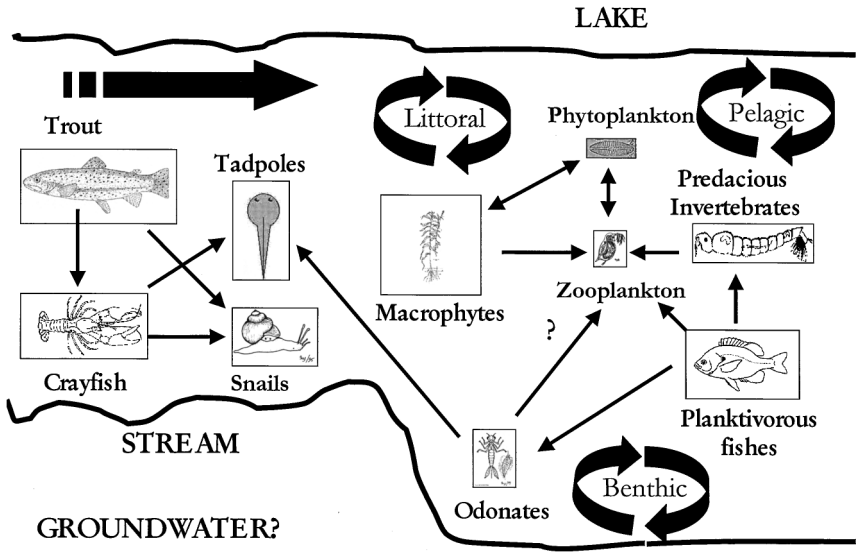


FIG. 3. Schematic of potential interactions mediated by chemical cues in either stream or lake habitats. Large arrows indicate direction of water flow. Curved large arrows suggest that water, and subsequently chemical cues, would circulate or be recycled. Smaller lines with arrows originate with the sender of the chemical cue and point to a potential receiver. Line drawings come mostly from BIODIDAC and a few noncopyrighted images from the Internet. Image of diatom can be found at Bowling Green State University's Algal Images.

RECENT ADVANCES

Quantitative Characterization of Chemical Cues

Probably every ecologist working on chemical ecology would like to know the exact chemical composition of the cue they have documented (Brönmark and Hansson, 2000). Knowledge of chemical structures would advance our insight into the metabolic origins, likely receptors, and the likely persistence and relative importance of different cues (Dodson et al., 1994). However, many ecologists are not trained in biochemistry and do not have the facilities, personnel, or funding necessary to investigate the identity of their chemical cue.

To explore how competition and predation regulate freshwater community structure, ecologists often refer to ecologically relevant chemical cues largely without knowing the chemical structure of the compounds in question. The ecologist places his/her focus on the result of the interaction that takes place when two or more organisms cooccur. A chemist, on the other hand, wants to know what specific chemical environment influenced the result of the interaction. Therefore,

scientists approach the same model system at different scales with different goals. Integrating these approaches, chemical identification with ecological results, is a sizable challenge.

Most compounds that freshwater ecologists suggest act as cues have not been isolated, purified, or structurally identified through complete biochemical assays. Only a few related to freshwater herbivory on macrophytes have been identified or quantified (Newman et al., 1996; Wilson et al., 1999; Kubanek et al., 2001), far fewer than analogous work in terrestrial and marine habitats. Instead, discussion of compounds in freshwater systems is generalized chemically, as groups of compounds (i.e., phenolics) or by chemical properties (lipophilicity, polarity, heat stability, etc . . .). Below we highlight interactions between organisms that show the greatest potential for complete characterization and identification of ecologically relevant chemical cues.

Phytoplankton–Zooplankton Interactions. Hessen and van Donk (1993) showed that the presence of the grazer *Daphnia magna* stimulated formation of large colonies in the green alga *Scenedesmus*. Exposure to *Daphnia*-cued water also increases the proportion of fatty acids that occur in *Scenedesmus* (Lürling et al., 1997), a possible mechanism to maintain buoyancy. These colonies are more difficult for daphnids to graze (Lürling et al., 1997) and, thus, potentially change the magnitude of top-down control of algae in lakes.

Von Elert and Franck (1999) characterized the active infochemical released by *D. magna* as an olefinic (i.e., an alkene with only one carbon–carbon double bond), low-molecular-weight (<500 Da) carboxylic acid. Furthermore, bioassays excluded hydroxyl and amino groups as fractions of the infochemical essential for biological activity (von Elert and Franck, 1999). Concentration of 1 liter of *Daphnia*-cued water using C-18 solid-phase extraction (C-18 SPE), followed by desorption with methanol (10 ml), evaporation, and later resuspension (100 ml methanol), produced similar colony formation as occurred when *Scenedesmus* was exposed to nonfractionated *Daphnia*-cued water (Lürling and von Elert, 2001). Collectively, these studies suggest that the daphnid infochemical responsible for colony formation is moderately lipophilic.

Other studies suggested that urea produced by *Daphnia* also induces colony formation in *Scenedesmus* (Wiltshire and Lampert, 1999). However, urea is a polar molecule without lipophilic portions, thus constituting a substantially different molecule from those tested in other bioassays. Consequently, debate continues as to which molecule, or combination of molecules, acts as an infochemical that induces colony formation in *Scenedesmus*. By separating out urea from a more lipophilic substance using C-18 SPE, Lürling and von Elert (2001) suggest that the colonizing-inducing activity of *Daphnia*-cued water is more lipophilic than urea-related. However, the specific identity of the structure(s) remains unresolved.

Stepping beyond *Daphnia* as the model of aquatic chemical communication, measurable quantities of heptadecene-1 exist within swarms of another

zooplankter, *Polyphemus pediculus* (Wendel and Jüttner, 1997). This volatile organic compound was identified using gas chromatography–mass spectroscopy (GC-MS) and may serve as a pheromone to maintain swarms. Wendel and Jüttner (1997) speculate that swarms may reduce mortality from predation. Heptadecene-1 is available as a pure compound, thereby encouraging further experiments on the ecological role of this chemical cue.

Fish Kairomones and Vertical Migration of Daphnia. Although we know that predators prompt diel vertical migration (DVM) of *Daphnia* and other zooplankters, we know relatively little about the chemical cues responsible for this well-studied phenomenon. Similar to other examples, ecologists can only speculate about the chemical nature of the cue(s), as specific molecules are still unknown. Bioassays suggest that the vertical migration-inducing kairomone in *Daphnia* is a freely dissolved compound of low molecular weight ($<$500 Da), with a lipophilic component, but no carboxyl-, phosphate-, or sulfate groups (von Elert and Loose, 1996). Amplitude of migration is also related to the concentration of this kairomone (von Elert and Pohnert, 2000), illustrating the importance of future tests of natural field concentrations.

Behaviorally or with life history strategies, *Daphnia* often do not distinguish between exposures to different fish species. Ecologists might hypothesize that these generalized responses indicate that kairomones from different species of fish are similar, if not identical (von Elert and Loose, 1996). However, chemists may question whether the active metabolites produced by these different fishes are the same, given that a structure has yet to be determined. This line of thought can only remain speculation until the chemical structures of these cues are actually characterized.

The most recent advancement with the zooplankton–fish system is one in which a hypothesis was rejected by follow-up studies. Boriss et al. (1999) proposed that trimethylamine (TMA) constituted the active component in fish cue, but subsequent investigations showed that daphnids responded only to concentrations of TMA higher than would naturally occur (Pohnert and von Elert, 2000). Furthermore, studies of life history responses of various daphnids to TMA have produced mixed results (Sakwińska, 2000; Lass et al., 2001). Not focusing on any specific compound, Ringelberg and Van Gool (1998) suggested that bacteria produced the kairomone responsible for inducing vertical migration, as antibiotic-treated perch-cued water suppressed migratory responses of *Daphnia*. However, treatment of fish-cued water with antibiotics did not eliminate migration by daphnids. Furthermore, antibiotics would only kill bacteria, not degrade a previously produced compound. Therefore, the origin and identity of the cue(s) responsible for inducing vertical migration remain a mystery.

Defense Compounds of Aquatic Macrophytes. Many freshwater macrophytes, like terrestrial plants and marine macroalgae, harbor chemical defenses (Lodge et al., 1998; Hay and Lodge, unpublished data). Only a few studies actually identify

the composition of deterrent secondary metabolites (Newman et al., 1996, Bolser et al., 1998, Kubanek et al., 2000). Perhaps more common are studies that speculate that unidentified macrophyte phenolics (benzene-ringed compounds with an —OH group), or subclasses of phenolics including tannins (phenolics that can precipitate proteins) or lignans (structural phenolics), are deterrents to herbivores (Lodge, 1991; Cronin, 1998; but see Dorn et al., 2001) and also to epiphytic algae (Gross et al., 1996).

It is important, however, to emphasize that not all phenolics or tannins are deterrent compounds. For example, Bolser et al. (1998) found that the crayfish *Procambarus clarkii* preferred macrophyte species both with high (*Typha angustifolia*) and low (*Ceratophyllum demersum*) phenolic concentrations, thus contradicting the hypothesis that phenolics always act as a deterrents to herbivory. To our knowledge, no study specifically isolates and identifies a freshwater tannin as a deterrent to herbivory. Furthermore, confusion may occur as to what compounds are being tested, depending on laboratory procedures. Colorimetric assays often used to quantify deterrent compounds do not discriminate among classes of molecules and instead capture total phenolics. Therefore, a danger exists in making generalizations about the deterrent properties of large, variable groups of compounds like phenolics.

Instead, the greatest advances in this field come from studies that have actually identified compounds responsible for deterring herbivory. For example, Newman et al. (1996) demonstrated that glucosinolate deterred multiple herbivores from consuming watercress (*Nasturtium officinale*). In addition, habenariol (bis-*p*-hydroxybenzyl-2-isobutylmalate) deters the crayfish *Procambarus clarkii* from feeding on the freshwater orchid *Habenaria repens* (Bolser et al., 1998; Wilson et al., 1999), and numerous lignoids deter this same crayfish from feeding on lizard's tail, *Saurus cernuus* (Kubanek et al., 2001). Besides influencing herbivory, specific plant compounds may affect competition between submerged macrophytes and phytoplankton. For example, *Myriophyllum spicatum* (Eurasian water milfoil) produces a hydrolyzable polyphenol, tellimangradin II, which deters algal growth (Gross et al., 1996). The possibility that allelopathic compounds play important roles in stabilizing alternative states of lake ecosystems—clear water macrophyte-dominated or turbid algal-dominated (Scheffer et al., 1993)—is an important hypothesis awaiting rigorous testing.

Trade-Offs and Multiple Cues

While identification of active compounds has been part of the advances described above, the multiplicity of cues in many freshwater environments requires that nonreductionist approaches be applied. Testing isolated compounds may often be a poor simulation of natural conditions, and, on practical grounds, we are unlikely to identify all the relevant compounds in the foreseeable future. For

example, extensive bioassays and chemical detective work led to the identification of bis-*p*-hydroxybenzyl-2-isobutylmalate (Bolser et al., 1998; Wilson et al., 1999) and several lignan compounds (Kubanek et al., 2000). However, in both cases, other deterrent metabolites in the water-soluble and lipid fractions remain unidentified (Hay, personal communication). Furthermore, the structural complexity of some molecules makes it difficult to identify pertinent structures, and small stereotypic changes in the molecule may enhance or negate the molecule's "activity" as a cue in communication. Therefore, while collaborating with chemists to identify compounds is recommended, progress in understanding the role of chemicals in community and ecosystem processes does not need to wait on complete chemical characterizations.

The influences of multiple chemical cues may not always be additive (Turner et al., 1999), and predictions based on exposure to only one cue at a time may be incorrect (Sih et al., 1998; Hazlett, 1999). Testing whole suites of chemical cues, in combination with single cue tests, may allow the researcher more power in extrapolation (Peckarsky et al., 1997). For example, a natural suite of (unidentified) littoral zone chemicals caused *Daphnia magna* to grow and reproduce more slowly than when grown in spring water (Burks et al., 2000). Laboratory experiments confirmed that daphnids altered their life history traits in response to cues from both macrophytes (waterweed, *Elodea canadensis*) and fish (roach, *Rutilus rutilus*). This result partially explains the paradox between historical studies documenting *Daphnia* avoidance of macrophytes, and recent studies illustrating that macrophytes can provide a refuge for *Daphnia* against predation by fishes (Burks et al., 2001). Without predation risk, daphnids avoid macrophytes because a reproductive cost comes with exposure. This cost may be outweighed by benefits of reduced mortality from fish predation in vegetated habitats.

Behavioral or life history trade-offs may require multiple chemical cues. For example, predator-induced diapause (production of ephippia) in *Daphnia magna* requires cues from both crushed-up conspecifics and kairomones from fish (Slusarcyzk, 1999). Therefore, organisms need to possess the ability to distinguish among multiple chemical cues. Dytiscid beetles (*Acilius sulcatus*) discriminate between chemical cues from hungry and satiated perch (*Perca fluviatilis*) and between perch satiated on fish versus those satiated on dytiscids (Åbjörnsson et al., 1997). Damselflies (*Enallagma sp.*) consistently decreased their movement and foraging in the presence of cues from crushed-up sympatric organisms (pike, *Esox lucius*, fed fathead minnows, *Pimephales promelas*). However, damselflies only responded to cues from biologically unrelated organisms (i.e., mealworms, *Tenebrio molitor*) with previous exposure to an actively foraging predator (Chivers et al., 1996). Even neurologically simple organisms such as flatworms (*Dugesia dorotocephala*) recognize fish odor as an indication of risk based on a single, simultaneous exposure to crushed conspecific and predator cues and do not respond to fish odor without the conspecific cue (Wisenden and Millard, 2001). Thus,

even apparently simple prey exhibit complex, chemically mediated responses to predators.

The presence of multiple predators may also elicit nonlinear, or unpredictable responses. When exposed to chemical cues from crayfish (*Orconectes rusticus*), physellid snails avoided covered habitats in contrast to seeking refuge when exposed to cues from pumpkinseed sunfish (*Lepomis gibbosus*). However, exposure to chemical cues from both predators produced an intermediate response (Turner et al., 1999, 2000), instead of the predicted response to the most dangerous predator. Identifying which cues are dominant controls in species interactions can be a challenging task. Trout cues suppress the impact of stonefly cues in inducing drift by stream-dwelling *Baetis* mayflies (Peckarsky and McIntosh, 1998), and cues from planktivorous fishes override the repellency of macrophytes in *Daphnia* (Lauridsen and Lodge, 1996; Burks et al., 2001).

Bullfrog tadpoles (*Rana catesbeiana*) exhibited nonadditive responses to multiple predators (i.e., dragonfly *Anax junuis* and bluegill *Lepomis macrochirus*), as only small tadpoles decreased their swimming with both predators and significantly more so with dragonflies (Eklöv, 2000). In testing responses of six larval anurans (wood frogs, leopard frogs, American toads, bull frogs, green frogs, and grey treefrogs) to five different predators (mudminnows, dragonfly larvae, newts or salamanders, dytiscid beetles, and belostomatid water bugs), Relyea (2001) found that the six prey species exhibited different responses to the same predator and that each prey species responded differently to the assorted predators. Clearly, the natural environment is full of predators and cues. Studies examining interactions among multiple cue effects will increase our understanding of the role of chemical ecology in structuring natural freshwater communities.

Abiotic Influences That Span Spatial and Temporal Scales

In many cases, the impact of chemical cues depends on the abiotic context, including light, temperature, and hydrodynamics. Chemical cues from light and predators are known to act in concert to prompt vertical migration of *Daphnia* (Brewer et al., 1999). *Chaoborus* only migrated toward light in the presence of chemical cues from fish occurring in combination with a moonlight gradient (O'Bryan and Forrester, 1997). More studies that identify proximate versus ultimate cues will add to our understanding of ecological interactions.

Temperature may also change the costs and benefits of responding to kairomones from predators (although see Sakwińska, 1998). For example, helmet size of *D. ambigua* reared with *Chaoborus* kairomones decreased with decreasing temperatures below 23°C (Hanazato, 1991). Furthermore, mortality of large helmeted daphnids increased at high temperatures (above 28°C). Thus, exposure to predator chemical cues reduced the tolerance of *Daphnia* to high water temperatures that commonly occur during summer.

Hydrodynamically different freshwater environments also influence the duration and properties of chemical cues. In streams, we generally assume that chemical cues originate upstream and influence taxa downstream. However, Dahl et al. (1998) demonstrated that *Gammarus pulex* uses chemical cues from downstream predators (trout) to assess drift behavior. Thus, eddies may play an important role in dispersing cues.

In freshwater systems, the best demonstration of the effect of flow involves crayfish foraging and orientation, analogous to the well-studied effects of flow and chemical cues on blue crabs and lobsters in marine systems (Zimmer and Butman, 2000). Crayfish (*Orconectes rusticus*) located food more quickly in streams with cobble (turbulent flow) versus sand substrate (more laminar flow) (Moore and Grills, 1999). Cobble breaks up the plume and increases dispersion, while laminar flow localizes the cue in parts of the stream not occupied by crayfish. The spatial distribution of complex odor cues (food + predator presence) also significantly impacted crayfish foraging (Tomba et al., 2001) when predator cue occurred in the middle of an artificial stream. Under this scenario, crayfish spent more time using side shelters and less time finding the food source. Thus, orientation to food and predator cues in changing flow regimes may strongly influence community interactions and ecosystem function. Experiments should test responses to chemical cues across a range of natural field conditions, including diverse spatial and temporal scales.

Studies that address larger spatial and temporal scales may discover patterns not apparent or predictable from extrapolation of smaller scales (Peckarsky et al., 1997). For example, McIntosh et al. (1999) pumped "trout-cued" water into a trout stream and monitored drift response of the mayfly, *Baetis bicaudatus.* Minimal drift occurred during the day in streams containg either background trout cues or added cues, but significantly less drift occurred at night in the stream where additional trout cues were added (McIntosh et al., 1999). Thus, mayflies quickly responded to the presence of trout cues. This group also added cued water into fishless streams and found that size at maturity of *Baetis* declined in streams where trout cues occured (Peckarsky et al., 2002). To test how quickly naive prey fishes respond to a predator, Brown et al. (1997) added juvenile pike to a 4-acre pond filled with fathead minnows. Minnows recognized chemical cues from pike after only 2 days, whereas fright responses to a visual cue did not occur for up to 8 days. Potential prey show differential responses to chemical cues, depending on experience. Lake-dwelling *Chaoborus* species (that coexisted with fish) exhibited no preference among ovipositing sites, while pond species showed an ovipositing preference for fish-free water (Berendonk, 1999). These results suggest that ovipositing behavior, influenced by chemical cues from fish, contributes to landscape-level patterns in *Chaoborus* distribution.

Looking for geographic patterns in predator avoidance, Covich et al. (1994) found that two species of physellid snails responded equally to crayfish taken from

their own habitat or from another geographic region. This result suggested that some generality exists in the crayfish kairomone. However, crawl-out responses of six snail species exposed to a single crayfish suggested species-specific responses that depended on microhabitat use (Covich et al., 1994). Thus, more studies investigating the generality of cues are needed to establish a contextual basis for prey response.

In addition to conducting research on large spatial scales, investigating time scales at which responses occur and cues last is also a topic ripe for study. De Meester and Cousyn (1997) suggest that behavioral responses of *Daphnia* to fish kairomones are rapid and reach their maximum after only a few hours. After exposure to chemical cues from fish, daphnids immediately sought refuge in macrophytes and maintained that response for at least 6 hours (Lauridsen and Lodge, 1996). Hazlett (1999) also found the behavioral effect on crayfish of predator odors from snapping turtles lasted only about 2 hours. Life history traits, on the other hand, may take longer to respond to chemical cues depending on genetic differences (see below) and may be enhanced in response to low food concentrations (Weber, 2001). Therefore, when addressing the generality of chemical cues, ecologists must explicitly consider the spatial and temporal scales relevant to the whole suite of chemical cues offered in a particular habitat or set of connected habitats. The relevance of chemical ecology to community and ecosystem ecology will be enhanced by larger and longer studies.

Chemical Communication within a Genetic Context

As the field of chemical ecology advances, studies not only need to identify important morphological and behavioral responses elicited by chemical cues, but also contrast phenotypic plasticity with underlying genetic variability. Increasing numbers of studies use *Daphnia* (which are parthenogenic most of the time) as a model to test the role of phenotypic and genetic variability and the broader evolutionary significance of chemically mediated responses (Lass and Spaak unpublished). Expression of ecologically relevant traits varies both within and between *Daphnia* populations (Stirling and Roff, 2000; for review, see De Meester, 1996a; Lass and Spaak unpublished). The presence of chemical cues from predators changes the selective pressures of local environments. For example, De Meester (1996b) measured the phototactic swimming behavior of ten *Daphnia* clones isolated from three different lakes (fishless, few fish, and numerous fish). Phenotypic plasticity differed among clones, with some altering their behavior more than others in the presence of fish. Furthermore, *Daphnia* clones from habitats with fish responded significantly more strongly to fish kairomones than daphnids from fishless systems (Boersma et al., 1999).

Genetic diversity may depend, in part, on the connectedness of the aquatic system. The self-contained nature of lentic systems may set boundaries for gene

flow and encourage local adaptation (De Meester, 1996a). However, for a lotic system, Storfer and Sih (1998) argue that gene flow between stream salamander populations (*Abystomas barbouri*) swamps local adaptation to chemical cues emanating from permanent streams with fish. Studies that begin to quantify the genetic variation associated with responses to chemical cues will add much insight to a growing field of interest.

OPPORTUNITIES AND CHALLENGES

Our review highlights several areas of freshwater chemical ecology poised for rapid progress. Research that identifies and quantifies chemical cues, such as the multifaceted approach used to characterize the *Daphnia–Scenedesmus* system, serves as a model for the study of inducible defenses (Tollrian and Harvell, 1999; van Donk et al., 1999; Lürling and van Donk, 2000). Recent identification of defensive compounds in freshwater macrophytes show the promise of this area to serve as a new arena in which to test theories of plant–herbivore interactions developed for terrestrial and marine systems. Further advances in freshwater inducible defenses, plant–herbivore interactions, and other areas will require greater collaboration of natural product chemists and ecologists to establish standard bioassays and chemical identification techniques that are practical and ecologically relevant.

Whether chemical cues are identified or not, much can be learned quickly with additional experiments using the natural suites of compounds existing in different habitats within lakes and streams. While cruder chemically, these sorts of experiments lead to rapid identification of larger scale patterns of trade-offs involving multiple cues. Identifying such patterns is essential to a more complete understanding of the organization of freshwater communities and the function of aquatic ecosystems. New studies should explore the importance of chemical communication in light-limited aquatic systems, such as groundwater or within caves. Such studies are relevant ecologically and provide rich material for subsequent chemical characterization.

In order to provide maximum insight into natural interactions, experiments must include realistic mimics of the complex abiotic milieu (flow, temperature, light, etc.) in which chemical cues are perceived by organisms (Moore et al., 2000). Comparisons of lake and stream environments may be especially fruitful. Freshwater environments provide excellent model organisms for the study of phenotypic and genetic responses to chemical cues and chemically mediated trophic interactions. Rapid progress can be made in this area, in a way that emphasizes the importance of chemical ecology to evolutionary ecology.

Overall, freshwater chemical ecology lags behind terrestrial and marine chemical ecology but provides vast immediate opportunities for interdisciplinary work of high relevance to chemistry, ecology, and evolution. Combining ecologists' desire to "assign ecologically relevant biological activity to natural products" with

chemists' desire to "unravel inter-organism communication at the molecular level" (Oldham and Boland, 1996) will lead to rapid progress of broad significance. These "ecologically relevant" interactions involve multiple species, vary within species, and take place within contextual environments dictated by flow, abiotic influences, and scale. Thus, questions in the field should increasingly attract other disciplines, including geneticists, physicists, and hydrologists. We must integrate these multiple perspectives and become "cued" into the diverse disciplinary cues.

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MINI REVIEW

MARINE TANNINS: THE IMPORTANCE OF A MECHANISTIC FRAMEWORK FOR PREDICTING ECOLOGICAL ROLES

THOMAS M. ARNOLD[1,*] and NANCY M. TARGETT[2]

[1]*Department of Biology*
University of Charleston
Charleston, South Carolina, 29424, USA

[2]*Graduate College of Marine Studies*
University of Delaware
Lewes, Delaware 19958, USA

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Abstract—Since chemical ecology emerged as a field of marine science, it has been strongly influenced by studies of chemically mediated interactions in land-based systems. Marine chemical ecologists, like their terrestrial counterparts, initially focused on identifying natural products and evaluating the potential ecological roles of these products as defenses, attractants, or other cues. Now, like our land-based colleagues, we must increase our focus on the physiological and biochemical mechanisms that underlie the chemical interactions, paying particular attention to regulation of biosynthetic pathways, within-plant and between-plant signaling cues, and comparative and functional genomics. Here, we review the current state of knowledge regarding a heterogenous group of macrophyte natural products, the marine tannins and simple phenolics, to illustrate how such information is critical to future attempts to predict their ecological roles.

Key Words—Marine chemical ecology, macrophytes, phenols, tannins, terrestrial – marine comparison.

INTRODUCTION

Plant tannins and related phenolic substances are common in marine ecosystems. They are produced by submerged vascular plants, emergent saltmarsh vegetation,

* To whom correspondence should be addressed. E-mail: arnoldt@cofc.edu

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and mangroves, as well as by brown algae. These reducing substances have primary roles in marine plants, serving as putative osmoregulatory substances in seagrasses and as components of cell walls in both marine vascular plants and brown algae (e.g., Schoenwaelder and Clayton, 1999). Phenolics also possess secondary roles. Many are antimicrobial agents, presumed to protect macrophytes against pathogen attack (Harrison and Chan, 1980; Vergeer and Develi, 1997), and some have also been identified as herbivore deterrents and herbivore digestion reducers, thought to increase macrophyte fitness by reducing herbivore pressure (e.g., Harrison, 1982; Boettcher and Targett, 1993; Pavia and Toth, 2000; but see Targett et al., 1995). Recent evidence also indicates that phenolics may protect macrophytes against harmful ultraviolet radiation (e.g., Pavia et al., 1997).

Traditionally, such discoveries have mirrored similar advances made for phenolic-rich plants on land, in part because marine ecologists have often borrowed biochemical techniques and ecological models of plant defense originating from terrestrial studies. More recently, however, advances in our understanding of phenolic production in marine plants have lagged, primarily because there have been few marine studies aimed at understanding the mechanisms of phenolic synthesis at the physiological, biochemical, and molecular levels. Dramatic variations in the phenolic contents of marine macophytes are commonly observed, but the regulation of phenolic production in marine macrophytes at the pathway or gene level has been largely unexplored. For example, the biosynthetic pathways for production of polyphenols in brown algae are still not known, despite the fact that polyphenols occur in most brown algal orders, can be present at concentrations as high as 25% of an alga's dry mass, and have been suggested to mediate numerous ecological interactions (Ragan and Glombitza, 1986; Targett and Arnold, 1998). Plant signaling molecules have not been well studied in marine macrophytes either, although they are known to be involved in the coordination of phenolic-based responses for terrestrial plants. As a result, we currently know little about the intrinsic and extrinsic factors regulating phenolic metabolism in these organisms. Yet this knowledge is crucial to our further understanding of how marine tannins and related phenolics influence plant–herbivore and plant–pathogen interactions in marine systems. The following review highlights the current state of knowledge regarding marine tannins and related phenolics and seeks to encourage interactions between terrestrial and marine chemical ecologists, especially in attempts to understand phenol production at the pathway and/or gene level.

MARINE VASCULAR PLANTS

Shikimate-Derived Phenolics

Phenolics derived from shikimate have long been associated with antipathogen and antiherbivore defense in land plants (Hammerschmidt and

Smith-Baker, 1999). These substances (and their oxidized products) have demonstrated roles as antimicrobial agents, herbivore deterrents/stimulants, digestion reducers, defense-related signaling molecules, and cell wall strengtheners (see Nicholson and Hammerschmidt, 1992). Concentrations of these shikimic acid and phenylpropenoid pathway products vary dramatically in organisms, and these variations have often been shown to affect the fitness of plants, including agriculturally important species.

The majority of plant phenolics, including simple phenolic acids, condensed and hydrolysable tannins, and lignins, among many others, are products of the shikimic acid (SA) and phenylpropenoid (PP) pathways (e.g., Singh et al., 1991). The SA pathway occurs in bacteria, fungi, and vascular plants and begins with the condensation of the carbohydrate intermediates erythrose-4-phosphate and phosphoenol pyruvate to generate chorismate, from which the aromatic amino acids phenylalanine, tyrosine, or tryptophan are produced (Herrmann, 1995). These amino acids can be allocated in vascular plants for either protein biosynthesis or, alternatively, for the production of polyphenolics via the PP pathway (Jones and Hartley, 1999). The production of phenylpropenoids (e.g., C_6–C_3 compounds) begins with the deamination of aromatic amino acids and results in the formation of cinnamic and *p*-coumaric acids, from which more complex phenolics arise (e.g., Seigler, 1998). As a result, significant fractions of a plant's carbon and nitrogen pools can be tied up in intermediates and end products of the SA/PP pathways.

The regulation of the SA/PP pathways has been well studied in land plants. It has been demonstrated, for example, that activities of the enzyme 3-deoxyarabino-heptulosonate-7-phosphate synthase (DAHPS), which determines the rate at which carbohydrate intermediates enter phenolic metabolism, are induced by wounding and pathogen attack in tomato, potato, parsely, and *Arabidopsis* (e.g., Muday and Herrmann, 1992). Furthermore, isoforms of the enzyme phenylalanine ammonia lyase (PAL), which directs the flow of amino acids away from protein synthesis and towards the PP pathway for the production of hydroxycinnnamic acids, are responsive to extrinsic factors. Not surprisingly, PAL activities have been linked to the production of flavonoids, condensed tannins, and lignins (Liu and McClure, 1995; Lavola et al., 2000). This trade-off between the production of aromatic amino acids and polyphenols is of particular interest to plant ecologists since it forms the foundation of several popular ecological models of plant defense (e.g., Bryant et al., 1983; Jones and Hartley, 1999). Marine vascular plants also produce significant, and varying, amounts of phenolic substances; however, unlike for terrestrial plants, there have been no previous studies of the SA/PP pathway enzymes, their regulation by environmental factors, or their roles in plant defense against pathogens in any marine vascular plant. As the following examples illustrate, our understanding of marine plant–herbivore and plant–pathogen interactions would benefit greatly from molecular and biochemical

studies of the SA/PP pathway, and its regulation by intrinsic and extrinsic factors.

Seagrass Phenolics

Seagrass beds are productive ecosystems that occur along the coasts of most continents (Macia, 2000). Fifty-eight seagrass species have been described. All share a similar basic anatomy: blades emerge from short shoots (i.e., ramets) that, in turn, arise from a continuous rhizome, or underground primary stem (Dawes, 1998). Seagrasses are a rich source of phenolic substances, including phenolic acids, sulfated phenolic acids, and flavones, condensed tannins, and lignins; but not hydrolyzable tannins. In this regard, the phenolic secondary chemistry of seagrasses is similar that of the terrestrial grasses from which they evolved. Despite this, there is currently no information regarding the regulation of the SA/PP pathway in these plants, and only a few attempts have been made to link seagrass secondary chemistry to plant fitness in the face of herbivory and pathogen attack.

Simple phenolic acids are abundant and account for as much as half of the measured phenolics in seagrasses (Vergeer et al., 1995; Vergeer and Develi, 1997). The array of phenolic acids produced by seagrasses is dependent upon the species, population, and tissue examined and may include caffeic acid, protocatechuic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, ferulic acid, vanillic acid, and gentistic acid, among others (McMillian et al., 1980; Quackenbush et al., 1986; Ravn et al., 1994). Phenolic acid conjugates are also common; for instance, McMillian et al. (1980) found sulfated conjugates in all 43 species they examined. Concentrations of phenolic acids vary dramatically. For example, concentrations of rosmarinic acid and caffeic acid in eelgrass, *Zostera marina*, varied seasonally, ranging from 0.4 to 19.2 mg/g Dry Mass (DM) (Harrison and Durance, 1989; Ravn et al., 1994). Seagrasses also accumulate significant levels of condensed tannins (i.e., proanthocyanins) in leaf and shoot tissues. For example, concentrations of 3–11% DM have been recorded in the leaves of turtlegrass, *Thallasia testudinum*, from the Gulf of Mexico and Caribbean Sea (Arnold and Tanner, data not shown), and recent studies suggest that proanthocyanin levels vary significantly over brief periods of time (Arnold and Tanner, data not shown). Lignin concentrations vary as well, from <1 to 5% DM, but remain somewhat lower than concentrations found in terrestrial grasses (see Dawes, 1998).

Additional evidence for variable phenolic contents in seagrasses is provided by studies using nonspecific measures of 'total' phenolics, i.e., Folin-reactive substances. While the Folin assays do not discriminate among the various classes of plant phenolics, they have revealed additional examples of varying phenolic contents in seagrasses (Harrison and Durance, 1989; Vergeer et al., 1995). Clearly, levels of simple and polymeric phenolics in seagrasses can be both high and highly variable.

The potential ecological roles of the phenolic acids present in seagrasses have not been studied extensively. There is evidence that phenolic acids, which arise early in the SA/PP pathway, have the potential to serve as antimicrobial agents, herbivore deterrents, and osmoregulatory agents (Harrison and Chan, 1980; Harrison, 1982; Ravn et al., 1989; Vergeer and Develi, 1997). The majority of these studies have been laboratory-based investigations, however, and it remains unclear how influential phenolic acids are in determining seagrass fitness in nature. Similarly, there is no evidence indicating that condensed tannins or lignins have roles in seagrasses defense against herbivores or pathogens. Below, we summarize the current state of our knowledge regarding the potential roles of seagrass phenolics in widely studied plant–herbivore and plant–pathogen interactions.

Herbivory. Sea urchins, fishes, green turtles, manatees and dugongs consume seagrass tissues, especially in tropical grassbeds. In addition, seagrasses harbor numerous species of mesograzers, which feed primarily on the epiphytes present on seagrass blades. Yet, overall rates of herbivory in seagrass beds are typically low; for instance, grazers remove 10–50% of seagrass primary production, compared to 60–97% of marine macroalgae production (Hay and Steinberg, 1992; Cebrian et al., 1998; Macia, 2000). A large portion of seagrass biomass is not consumed by herbivores but rather is recycled as detritus, especially in temperate ecosystems (Zieman and Zieman, 1989). However, dramatic examples of grazing have been observed, usually in tropical ecosystems (Valentine and Heck, 1991; Greenway, 1995). For example, urchins, the predominant grazer on tropical species, have been observed to denude seagrass beds in the Caribbean Sea, Gulf of Mexico, Mediterranean Sea, and Gulf of Aqaba, as well as along the coasts of Australia and Japan (see Heck and Valentine, 1995). Seagrasses respond to, and are affected by, grazers. Herbivory can decrease primary production in cases where it results in the loss of photosynthetic tissues (Valentine and Heck, 1991; Cebrian et al., 1996); it may increase production where it removes leaf epiphytes (Thayer et al., 1984); it may, depending upon the resource status of plants, affect the growth rates of leaves and shoots (Cebrian et al., 1998). Plant phenolic metabolism may also be affected. Wound-induced production of condensed tannins and total reactive phenolics has been observed in several populations of turtlegrass, *T. testudinum*, subjected to simulated herbivory (Arnold and Tanner, data not shown). Herbivores, in turn, can also be affected by characteristics of the seagrasses they consume. Valentine and Heck (2001) demonstrated that changing nitrogen contents of seagrass tissues influenced grazing by the urchin *Lytechinus variegatus.* In this study, the herbivores grazing in unfertilized grassbeds compensated for the lower nutritional quality by increasing consumption compared to urchins grazing on N-rich plants from fertilized grassbeds.

The potential for seagrass phenolics to serve as chemical defenses against herbivores, while intriguing, has been rarely considered (but see Harrison, 1982). For example, while it seems reasonable to expect these vascular plants to respond

to herbivores, it remains to be seen if (or how) real herbivory induces altered phenolic contents as artificial wounding does. Future studies examining the response of pathway enzymes and gene expression would be helpful, for while plants often respond at these levels immediately, it typically takes a period of days or weeks before grazer-induced accumulations of phenolics can be detected. Moreover, since the potential for phenolics to serve as defensive agents can be influenced by oxidation (see Appel, 1993), it would be worth examining activities of polyphenol oxidase enzymes or the expression of related genes sequences as well. Finally, given the clonal nature of seagrasses, it is worth exploring the possibility of a systemic response, resulting from the translocation of chemical signals via the vascular system.

Disease Resistance. Evidence also exists to support a role for phenolics in seagrass disease resistance, particularly with regard to the wasting disease that contributes to rapid population declines for certain species. This disease has been implicated in the mass mortalities of eelgrass occurring in North America, Europe, and New Zealand, as well as the continuing decline of turtlegrass in Florida Bay (USA) (Short et al., 1986; Duranko and Kuss, 1994). It causes expanding necrotic lesions on blades and shoots and leads to mortality of a large percentage of affected plants (Muehlstein, 1992; Den Hartog, 1996). Disease outbreaks have long-lasting effects on seagrass populations; for example, many eelgrass populations affected by the disease in the 1930s have not fully recovered, and some were driven to local extinction (see Short et al., 1986; Muehlstein et al., 1988). The pathogenic organism infecting eelgrass is *Labyrinthula zosterae* Porter et Muehlstein, a net-forming protist of the phylum Labyrinthulomycota. A related species is associated with turtlegrass, *T. testudinum* (Duranko and Kuss, 1994; Zieman et al., 1999). *Labrinthula* spp. are nearly ubiquitous in coastal marine ecosystems and only periodically, for unknown reasons, do they become virulent pathogens (Muehlstein, 1992; Vergeer and Den Hartog, 1994). It has been observed, however, that the emergence of the disease is often preceded by declines in plant health linked to a variety of changing environmental conditions, such as decreased light levels, elevated temperatures, and high salinities (as reviewed in Den Hartog, 1996). Disease resistance has also been linked to levels of plant phenolics. Specifically, the phenolic content of eelgrass leaves and shoots has been correlated with resistance to the wasting disease in mesocosm experiments (Buchsbaum et al., 1990), and seagrass phenolics possess growth-inhibiting activity against *Labyrinthula* spp. grown in culture (Vergeer and Develi, 1997). Moreover, these putative phenolic defenses are inducible. Levels of both total reactive phenolics and caffeic acid can be induced by *Labyrinthula* sp. attack (Vergeer et al., 1995).

An understanding of the mechanisms of seagrass–pathogen interactions could be helpful in explaining and/or predicting seagrass die-offs. For example, it is interesting to note that many of the environmental factors linked to wasting disease

outbreaks are known to inhibit the SA/PP pathway in land plants. Decreased light levels and elevated nutrient concentrations, two hallmarks of coastal degradation previously suggested as triggers of wasting disease outbreaks, inhibit enzymes of these pathways in land plants. If seagrass phenolics do have a role in determining disease resistance and if the SA/PP pathway of seagrasses is affected by coastal eutrophication, then this may help to explain the timing of past seagrass die-offs or, potentially, serve as a bioindicator of future events.

Emergent Plants

Phenolic-rich vascular plants also occur in tidal plant communities, including salt marshes and mangrove forests. Salt marshes occur over a wide range of latitudes, from arctic to subtropical habitats and are composed of a variety of emergent species, including grasses, herbs, and shrubs. Mangrove forests are tropical communities, which are dominated by several species of mangals. Both types of communities act to stabilize coastal sediments, contribute to land formation and serve as nursery habitats. Both have suffered from overexploitation.

Salt marsh plants accumulate relatively high levels of phenolic substances that may protect them from herbivores and that leach, as so-called humic substances, into nearby waters (e.g., Filip and Alberts, 1989). Rates of herbivory on salt marsh plants are generally low; however, grazing is not homogeneous. Grazing by phytophagous insects, migratory waterfowl, and marine invertebrates can vary, and there is some evidence that plant phenolics are influential in these interactions (e.g., Buchsbaum et al., 1984). For example, growth of the salt marsh periwinkle, *Littoraria irrorata*, on *Spartina alterniflora*-based diets has been negatively correlated with the phenolic content of the food (Baerlocher and Newell, 1994), and the phenolic content of sawgrass leaves in the Florida everglades has been linked to rates of herbivory and fungal infection. In the latter case, the phosphorus-enriched plants had lower phenolic contents and higher rates of grazing and infection (Richardson et al., 1999). These examples provide support for the view that phenolics influence grazing; however, many other plant characteristics also contribute to the observed patterns of herbivory (e.g., see Pennings et al., 2001). In mangroves, tannin concentrations are reported to range from 8.8 to 40.8% DM, and levels of total reactive phenolics vary from 11.1% to 48.8% DM (Basak et al., 1996, 1998). These levels are high enough that the dried bark of certain mangrove species was used to tan leather (Dawes, 1998). The characteristic blackened prop roots of these plants are the result of phenolic exudates that react with ferric ions from the soil solution (Kimura and Wada, 1989). Variations in tannin contents and total phenolic levels occur. For example, Feller (1996) provided evidence for increased phenolic contents in leaves of phosphorus-enriched mangroves. Other variations in phenolic content have been negatively correlated with plant protein levels in some studies (Basak et al., 1998), but not others (Basak et al.,

1996). Such variations are likely to have ecological significance. Phenolics from mangroves act to modify sediments near prop roots (Kimura and Wada, 1989) and may negatively affect meiofaunal species in mangrove estuaries (Alongi, 1987). In addition, they are predicted to influence rates of decomposition in mangrove forests. For example, a field study aimed at assessing the effect of nutrient enrichment on stands of dwarf red mangrove *(Rhizophora mangle*) indicated that leaf phenolic contents were linked to rates of decomposition in belowground sediments, but not in aboveground leaf litter (see Feller, 1996). Attempts have been made to correlate phenolic contents with herbivory but these have yielded mixed results. In a study of 10 mangrove species in India, herbivore damage was negatively correlated with leaf tannin contents (Kathiresan, 1992). While the leaf-eating mangrove crabs *Sesarma messa* and *S. smithii* from Australia have been observed to prefer tannin-free over tannin-rich leaves (Camilleri, 1989; Steinke et al., 1993), feeding by the congener *Sesarma meinerti* from South Africa is unaffected by leaf tannin levels (Micheli, 1993). The balance of data to date suggests that phenolics act as one of many factors influencing litter decomposition and herbivory.

Like the case illustrated for seagrasses previously, there have been few studies of salt marsh plant phenolic metabolism, especially at the molecular and/or biochemical levels. Future studies of this sort are likely to aid in efforts to: (1) understand how increases in atmospheric CO_2 concentrations are likely to affect the phenolic content of marsh grasses and whether or not these would influence rates of organic matter recycling in wetlands (e.g., Wilson et al., 1986), (2) better predict the outcome of plant–herbivore interactions, including those associated with biocontrol of invasive wetland plants (see Wecker et al., 2000), and (3) evaluate the potential affects of coastal eutrophication on the secondary chemistry of these plant communities.

MARINE NONVASCULAR PLANTS

Acetate-Derived Tannins

Polyacetate-derived phenolics occur in a wide range of terrestrial and aquatic organisms, including algae, bacteria, fungi, lichens, and vascular plants. Many are bioactive and have roles as toxins, antibiotics, immunosuppresants, antitumor agents, and antiparasitic agents. Well-known examples include: aflatoxin, a potent cytotoxin and carcinogen produced by certain fungal species; erythromycin, a commonly prescribed antibiotic from *Streptomyces* sp.; urushiols, skin irratants produced by poison ivy, *Toxicoderndron radicans*; and the polyacetates from the fungus *Ceratocystis ulmi*, which contribute to the symptoms of Dutch elm disease (Seigler, 1998 and references therein). Polyacetates (i.e., polyketides) are

generated via the condensation of acetate and malonate units, in a manner similar to the synthesis of fatty acids (Packter, 1980). The sequential condensation of acetate and malonate units is catalyzed by polyketide synthase (PKS) enzyme complexes. Thousands of polyacetate natural products arise as variations on this basic metabolic theme, and their production is considered to be an example of combinational chemistry (Reynolds, 1998). Interestingly, some phenolics of vascular plants are generated using both PKS enzyme complexes and the aforementioned SA/PP pathway. These arise from the precursors chalcone and 6′-deoxychalcone, SA/PP pathway intermediates that are generated by the enzyme chalcone synthase, a plant-specific polyketide synthase.

Brown Algal Phenolics

In marine systems, the most widely studied polyacetate phenolics are the phlorotannins of marine brown algae. Brown algal phenolics are sequestered in cellular microvesicles called physodes (Ragan and Craigie, 1976) or, alternatively, are incorporated in brown algal cell walls (Schoenwaelder and Clayton, 1999). Phlorotannins are similar to the condensed tannins of vascular plants with regard to their basic chemical properties and observed ecological roles. For instance, reduced phlorotannins bind to metal ions and a variety of macromolecules, including proteins, carbohydrates, and nucleic acids and may function as cell wall components, putative spermatozoid inhibitors, UV sunscreens, antibacterial agents, herbivore deterrents, and digestion inhibitors (for review see Ragan and Glombitza, 1986; Stern et al., 1996; Targett and Arnold, 1998). However, unlike the condensed tannins, phlorotannins are not products of the SA/PP pathway. They are believed to arise from the condensation of acetate and malonate units via a polyketide synthase (PKS) type enzyme complex although, to date, no such complex has been described in brown algae.

Phlorotannin levels vary dramatically. For example, in macrophytes from the temperate and tropical Atlantic, northeastern Pacific, and Antarctic Oceans, concentrations of 5–12% DM are common; however, tropical Indo-Pacific populations possess uniformly low levels (<2% DM) (Ragan and Glombitza, 1986; Steinberg and van Altena, 1992; Targett et al., 1992, 1995). Regional variations also occur and have been linked to macrophyte age, algal size, irradiance levels, nutrient availability, herbivore densities, and season (Ragan and Glombitza, 1986; Van Alstyne et al. 2001). Such variation is one reason why algal phlorotannins have been so often used in tests of plant defense theory in marine systems: the degree to which phlorotannin levels vary in nature is similar to that observed for tannins in vascular plants. Tests of these ecological theories using phlorotanin-rich algae as model systems have been numerous, examining macrophytes in both the field and in culture. However, the majority of studies have been correlative and,

to date, no ecological theory or model has emerged as an acceptable predictor of algal phlorotannin levels (see Cronin, 2001). At a larger scale, the absence of phlorotannin-rich algae in the tropical Indo-Pacific has not been explained. Recently, both the effectiveness of phlorotannins as herbivore deterrents and their classification as secondary metabolites have been questioned.

We suggest that these and other debates regarding the roles of brown algal phlorotannins would benefit from evaluations of brown algal physiology. For instance, some of the difficulties in testing the assumptions and predictions of these ecological theories are due, in part, to the lack of pathway- and gene-level information regarding phlorotannin production, as the following examples illustrate.

Carbon–Nutrient Balance Hypothesis (CNBH). This hypothesis originated from studies of woody plants in boreal forests and proposes that plant phenolic contents may vary with regard to resource availabilities (see Bryant et al., 1983). According to the CNBH, such links arise because carbon-based defenses (e.g., polyphenolics and terpenoids) accumulate when amino acid biosynthesis is restricted by nitrogen limitation. Such a response is hypothesized to improve plant fitness since carbon-based defenses would then accumulate under conditions when nitrogen-rich tissues would be most difficult to replace (Bryant et al., 1983). Tests of this model have examined carbon-based defenses from a wide range of terrestrial plant species as well as plant cell cultures (Kubek and Shuler, 1980) and marine macrophytes (Buchsbaum et al., 1990). The CNBH has been commonly used in attempts to account for variations in brown algal phlorotannin contents, with varying success (see Pavia and Brock, 2000 and references therein; Cronin, 2001).

In recent years, the CNBH theory has become increasingly criticized (see Hamilton et al., 2002). While some carbon-based compounds (e.g., phenolics) are often observed to vary as predicted by the CNBH, many other compound types (e.g., terpenoids) do not seem to follow CNBH prediction (Cronin, 2001). Differences among various classes of carbon-based defenses may be the result of differential regulation of the associated biosynthetic pathways or differences in product turnover rates and, therefore, costs (Cronin, 2001). Terrestrial chemical ecologists have also grown increasingly frustrated by the lack of a mechanistic basis for the CNBH, which does not indicate exactly how its predicted trade-offs may be generated in plant cells. As a result, other, more mechanistic models have been proposed in an attempt to link variations in plant phenolic contents with environmental stressers via specific metabolic mechanisms. For example, the protein competition model of Jones and Hartley (1999) shares many of the predictions also made by the CNBH but bases these predictions on a clear understanding of the SA/PP pathway, particularly the role of PAL in generating trade-offs between the production of aromatic amino acids and hydroxycinnamic acids. Its firm foundation in plant metabolism makes its specific assumptions more testable than those of the CNBH.

In retrospect, it is interesting to consider the use of the CNBH in past studies of brown algal phlorotannins, especially since recent observations suggest that this model should *not* apply to these compounds. For example, the CNBH is presumed to be effective in predicting variations of immobile defenses, i.e., those with low rates of turnover; however, recent evidence suggests phlorotannin turnover rates, like some vascular plant tannins, are relatively high (Arnold and Targett, 2000). Furthermore, the model is not expected to accurately predict variations for compounds that, like phlorotannins, possess multiple secondary roles and/or a combination of both secondary and primary roles (Arnold and Targett, 2002). In addition, there is no known metabolic mechanism in phlorotannin biosynthesis that would result in a trade-off between phlorotannin production and protein synthesis, like that resulting from the deamination of amino acids in the SA/PP pathway of vascular plants. This leads to the intriguing question of why intraspecific variations in phlorotannin levels are sometimes consistent with the predictions of the CNBH.

Induction. In some brown algae, accumulations of phlorotannins can be induced by herbivore grazing and simulated herbivory (Van Alstyne, 1988, 1989; Hammerstrom et al., 1998; Toth and Pavia, 2000), as well as by the presence of waterborne cues from grazing herbivores (Toth and Pavia, 2000) and the presence of airborne methyl jasmonate (Arnold et al., 2001). The response has been suggested to increase plant fitness, and there is indirect evidence supporting this stance. For instance, grazing by littorine snails is discouraged by the development of this induced response in the intertidal algal, *Fucus distichus* (= *F. gardneri*) (Van Alstyne, 1990). In addition to site-specific accumulations of polyphenols, plant-wide responses to grazing have been observed. In *Fucus* sp., for example, elevated polyphenol levels may propagate to undamaged fronds of the same plant, and grazing can result in the initiation of adventious branching as well (Van Alstyne, 1988, 1990). However, the ways in which these macrophytes perceive damage and coordinate a defensive response remain a mystery. Ironically, the presence of the phlorotannins themselves have impeded efforts to characterize enzymes and/or genes associated with this induced response, as they typically interfere with nucleic acid extractions. Nevertheless, recent studies of induced responses are beginning to reveal some intriguing clues. For example, oligoguluronates have been demonstrated to activate an oxidative burst in kelps (Collen and Davison, 1999; Küpper et al., 2001), and polyphenol induction can be activated by herbivore-specific cues (Pavia and Toth, 2000) and the presence of airborne methyl jasmonate (Arnold et al., 2001). Salicylic acid, a phenolic compound involved in the systemic responses of vascular plants, has been identified in brown algae (Legaz et al., 1985), but its roles in algal defense, if any, have not been examined. Certainly, when compared to what is known regarding signal transduction and pathway regulation in land plants, it is clear that our understanding of induced phenolic metabolism in brown algae is in its infancy.

SUMMARY

As these case studies illustrate, although marine tannins and related phenolic substances are common in marine macrophytes, we know very little about the physiological mechanisms controlling (poly)phenol metabolism in these organisms. As a result, marine macrophytes are generally treated as black (or green or brown) boxes. A better understanding of the biosynthetic pathways and key regulatory steps leading to phenol production in marine macrophytes would help us to predict variations in phenolic concentration in response to specific biotic and abiotic factors, understand local and systemic responses to herbivores, generate hypotheses regarding the evolution of defense strategies in marine macrophytes, and rigorously test ecological theories in marine ecosystems.

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MINI REVIEW

CHEMICAL CUES FOR SURFACE COLONIZATION

PETER D. STEINBERG,[1,2] ROCKY DE NYS,[3] and STAFFAN KJELLEBERG[2,4]

[1]*School of Biological, Earth, and Environmental Science*

[2]*Centre for Marine Biofouling and Bio-Innovation*

[3]*School of Biotechnology and Biomolecular Science*
University of New South Wales
Sydney, New South Wales 2052 Australia

[4]*School of Marine Biology & Aquaculture*
James Cook University
Townsville, QLD 4811 Australia

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Abstract—Colonization of surfaces in marine benthic environments is often one of the most significant moments in the life history of benthic organisms, representing, for example, a change from a planktonic to a benthic existence, a shift from a mobile to a sessile life form, or the initiation of pathogenesis. Many of the surfaces that are colonized are, in fact, other marine organisms, and in a general sense there is widespread evidence that specific chemical cues derived from marine organisms affect colonization by both marine prokaryotes and eukaryotes. However, detailed information for any one system on the nature of such cues, their distribution in situ, and their effects on the demography of colonizers is rare. Here, we selectively review the literature on chemical cues for colonization in the sea, focussing on contrasts between positive (inducers) and negative (inhibitors, deterrents) cues and on prokaryote/eukaryote interactions. We also consider whether generalized life history or natural history characteristics of colonizers (i.e., the mobility of a propagule, the extent to which a species is a habitat generalist or specialist, etc.) affect their response to chemical cues, and we touch briefly on some recent highlights relevant to the critical interplay between hydrodynamics and chemistry. A number of important methodological concerns are now being addressed through the introduction of field assays and analyses for chemical cues, and through molecular techniques for the characterization of

* To whom correspondence should be addressed. E-mail: p.steinberg@unsw.edu.au

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microbial biofilms. These developments are encouraging, as is the increasingly multidisciplinary and cross-taxonomic approach to research in this area.

Key Words—fouling; larval inducers biofilms; infection; chemical signalling.

INTRODUCTION

Most marine benthic organisms spend some portion of their existence—hours to many months, depending on the taxa—in the water column as larvae, spores, or other planktonic forms. The transition from a planktonic to a benthic existence is a fundamental change in the life history of benthic organisms and, by definition, requires colonization of a surface. These surfaces may be living (other marine organisms) or inanimate (primary substrata), although given the ubiquitous presence of microbial biofilms in marine systems (Wieczorek and Todd, 1998; Holmström and Kjelleberg, 2000), truly "inanimate" surfaces are probably rare.

As with other interactions among marine organisms, colonization of surfaces in the sea can be chemically mediated, with naturally produced metabolites acting as both positive and negative cues. Although we know that such cues can range from general properties of water chemistry such as salinity (Welch and Forward, 2001) to specific organic metabolites (Tsukamoto et al., 1999), the exact nature of the cues, their distribution in the habitat, and their importance for the demography of the interacting organisms is poorly known for all but a few systems. This contrasts with chemically mediated marine consumer–prey interactions, where a number of metabolites have been characterized, quantified, and shown to have strong effects on the behavior, demography, or community properties of consumers or their prey (see reviews in McClintock and Baker, 2001).

There are a number of recent reviews on chemical cues for colonization that together provide a comprehensive background for the subject (Fusetani, 1997; Slattery, 1997; Wieczorek and Todd, 1998; Fingerman et al., 1999; Holmström and Kjelleberg, 2000; Zimmer and Butman, 2000; Hadfield and Paul, 2001; Rittschof, 2001; Steinberg et al., 2001). Even a condensed overview of the topic as a whole is not practical here and, thus, we focus on a few general issues in the field that we believe are topical and important, while emphasizing the diversity of interactions that fall within the topic. Our focus will be on organisms as producers of naturally occurring cues as well as responders, in keeping with this volume's theme of interactions among organisms. Finally, we use the term "colonization" here in a broad sense, recognizing that the term can encompass a variety of different processes.

INDUCERS VERSUS INHIBITORS

Chemical cues can either enhance (inducers) or inhibit (inhibitors, "natural antifoulants") colonization. Most inducers of colonization appear to be primary metabolites such as sugars and/or carbohydrates or amino acids, peptides, and/or

proteins. They are also often water soluble or, if larger insoluble macromolecules, have water-soluble components that also function as cues. For example, small naturally occurring peptides induce settlement or metamorphosis of larvae of abalone (Morse, 1992; Roberts, 2001), oysters (Zimmer-Faust and Tamburri, 1994), sand dollars (Burke, 1984), nudibranchs (Lambert et al., 1997), and jellyfish (Fleck et al., 1999). Larger insoluble proteins induce settlement of barnacles (Clare and Matsumura, 2000). Carbohydrates induce settlement of corals (Morse and Morse, 1991, 1996), sea slugs (Krug and Manzi, 1999), and sea urchins (*Holopneustes purpurascens*) (Williamson et al., 2000), although an additional algal derived cue also induces metamorphosis of *H. purpurascens* (R. Swanson et al., unpublished). Partially characterized inducers of invertebrate settlement from bacterial biofilms are also frequently carbohydrates (Kirchman et al., 1982; Szewzyk et al., 1991). For both carbohydrates and proteins, the extent to which the active component of the cue is insoluble and surface bound versus released and water soluble is not always clear, and both may be present for inducers of corals (Morse and Morse, 1991), abalone (Morse, 1992), and barnacles (Clare and Matsumura, 2000). Glycoglycerolipids, compounds of mixed carbohydrate–fatty acid biosynthesis, from the green alga *Ulvella lens* induce settlement and metamorphosis of sea urchins (Takahashi et al., 2002). Fatty acids alone may also act as naturally occurring inducers of larval settlement, although the best known example of fatty acid inducers, for the tube worm *Phragmatopoma lapidosa californica* (Pawlik and Faulkner, 1986; Pawlik, 1990), has been challenged (Jensen and Morse, 1990; Jensen et al., 1990). One of the few fully characterized inducers, lumichrome from the ascidian *Halocynthia roretzi*, is a derivative of riboflavin (Tsukamoto et al., 1999).

A few inducers do not fit this general pattern of primary metabolites. The nonpolar secondary metabolite haliclonacyclamine from the sponge *Haliclona* sp. 628 induces settlement, but not metamorphosis, of the ascidian *Herdmania curvata* (Green et al., 2002) and, thus, is an example of a metabolite that acts on only one of the cascade of processes involved in colonization. A more general exception may be the nonpolar bacterial signal molecules acylated homoserine lactones (AHLs), the inducers in regulatory pathways, which control a wide range of colonization-relevant phenotypes in gram-negative bacteria (reviewed by Dunny and Winans, 1999; Whitehead et al., 2001; see below).

In contrast to inducers, deterrents appear to be primarily nonpolar secondary metabolites (Steinberg et al., 2001). Better known examples include halogenated furanones from the red alga *Delisea pulchra* (de Nys et al., 1995; Dworjanyn et al., 1999; Dworjanyn, 2001), terpenoids from the brown alga *Dictyota menstrualis* (Schmitt et al., 1995), and a variety of metabolites from sponges (Walker et al., 1985; Kubanek et al., 2002; Faimali et al., 2002). A much wider array of nonpolar metabolites from marine eukaryotes, when extracted from whole organisms, also deter colonization (Clare, 1996; Rittschof, 2001), but it is mostly unclear whether these compounds are active at surfaces in situ (de Nys and Steinberg, 1999).

Several explanations have been proposed for the differences in the nature of inducers versus deterrents (Rittschof, 1990; Steinberg et al., 2001). First, selection pressures acting on deterrents are different. Selection for deterrents should act most strongly on the producing organism, which needs to deter a broad suite of typically generalist (Wahl and Mark, 1999) colonizers that will usually have alternative sites for settlement. Selection for inducers should act primarily on the receiving organism to maximize its ability to detect and respond to cues, regardless of whether those cues are passively or actively produced.

Second, deterrents need act only at the surface of the producer, which is facilitated by metabolites that associate with the organic surfaces of producing organisms and have low solubility in seawater. In contrast, the inducers should be detectable from as far away as possible, which will be facilitated by water-soluble metabolites that often "leak" from the producing organism. This may explain why large insoluble inducers derived from, for example, cell walls often appear to have associated water soluble cues, which are either separate metabolites or dissociable components of the larger molecule (Morse and Morse, 1991; Krug and Manzi, 1999; Clare and Matsumura, 2000).

Third, given the potential for rapid dilution of cues once in the water column, inducers should be present in as high a concentration as possible. Individual non-polar secondary metabolites are typically produced at $<1\%$ of the dry weight of an organism, and as surface components they may be present at much lower levels (Dworjanyn et al., 1999). Primary metabolites—particularly carbohydrates—in marine organisms can be present at much higher concentrations.

Finally, organisms should be preadapted to use primary metabolites as inducers. Many marine organisms can absorb nutrients—simple sugars, amino acids—directly from the environment, and can respond to concentration gradients of these nutrients *via* chemotaxis (Amsler and Iken, 2001). In such instances, the nutrients (primary metabolites) function directly as signals. Nutrients in aquatic systems often accumulate at surfaces and, thus, provide a strong cue for the presence of surfaces even when no longer directly utilized as food. Many organisms also use primary metabolites—particularly peptides and amino acids or their derivatives—as components of internal signal transduction systems (Rittschof, 1990; Slattery, 1997). Thus, the receptor machinery for responding to similar but externally derived signals is already available to many taxa.

Methodological Issues: Characterization, Localization, and Quantification of Cues

The actual indentity of cues for colonization, whether they are present at or near a surface, and, if so, at what concentrations is known for remarkably few metabolites, and the issues for deterrents versus inducers again appear different in this respect. Because many inducers are small water-soluble molecules present against a background of similar molecules or complex macromolecules that may

be components of cellular skeletons, they have proven hard to characterize (Krug and Manzi, 1999; Clare and Matsumura, 2000; Hadfield and Paul, 2001). There are few source-specific, ecologically relevant inducers of colonization for which chemical structures are known. Examples include lumichrome from the ascidian *Halocynthia roretzi* (Tsukamoto et al., 1999), the floridoside–isethionic complex from red algae (Williamson et al., 2000), haliclonacyclamine from the ascidian *Herdmania curvata* (Green et al., 2002), glycoglycerolipids from the green alga *Ulvella lens* (Takahashi et al., 2002), and bacterial acylated homoserine lactones (Dunny and Winans, 1999).

In contrast, because most known deterrents are small nonpolar metabolites, they are amenable to the standard techniques of natural products chemistry. For deterrents, the primary issue has been whether the metabolites are present at or near the surface of the organism at biologically effective concentrations (Clare, 1996; de Nys and Steinberg, 1999). Examples of deterrents that have been localized and quantified at or near the surface are relatively few and mostly exist for seaweeds or sponges. Furanones from the red alga *Delisea pulchra* are encapsulated in vesicles in gland cells that are present at the surface (Dworjanyn et al., 1999) and release the compounds to the surface at concentrations what deter common epibiota (de Nys et al., 1995, 1998; Maximilien et al., 1998). Terpenoids in natural concentrations of surface extracts from the brown seaweed *Dictyota menstrualis* deter settlement of the bryozoan *Bugula neretina* (Schmitt et al., 1995). In contrast, other algal metabolites either do not appear to be present on the surface (de Nys et al., 1998) or if released at the surface are not present at sufficiently high concentrations (Jennings and Steinberg, 1997). Information on the localization of sponge secondary metabolites is rapidly increasing (e.g., Salomon et al., 2001; Faimali et al., 2002, Kubanek et al., 2002), and some of these metabolites are present at the (often morphologically complex) surface of sponges. Quantification of sponge deterrents is still relatively rare (Kubanek et al., 2002), but includes some of the earliest work on this topic (Walker et al., 1985). For bacterial biofilms, concentrations of inducers have been shown to vary dramatically on very small scales. Charlton et al. (2000) found that concentrations of acylated homoserine lactones (AHLs) in biofilms in flow cells were 3–4 orders of magnitude higher than in the bulk fluid millimeters above the biofilm. A variety of both high-tech [flow cytometry, immunolabeling, epiflourescence, and confocal laser microscopy, (XPS)] and low tech (surface swabbing and extraction, analysis of water samples collected at specified distances from a source) are now available for investigating and quantifying chemical cues at surfaces.

PROKARYOTE–EUKARYOTE INTERACTIONS

Marine prokaryotes: (1) communicate among themselves during the establishment of surface communities (such as in the formation of microbial biofilms), (2)

as colonists respond to eukaryotic chemical cues, and (3) are, in return, important as producers of cues for the settlement of eukaryote propagules.

Research on chemical communication among bacteria as a modulater of colonization of surfaces is a growth industry, but only recently is it intersecting with analogous studies for marine eukaryotes. Much of this work focuses on bacterial cell–cell signaling (reviewed by Dunny and Winans, 1999; Whitehead et al., 2001; Winans and Bassler, 2002), also known as quorum sensing because of its importance in high-density bacterial populations. The two most widely known examples of bacterial signaling are the acylated homoserine lactone (AHL) and autoinducer 2 (AI-2) systems. The details of the two systems differ (as do aspects of each system among different bacteria), but each is based on small signaling molecules (AHLs or the recently identified AI-2 signal, respectively) that diffuse between and into bacterial cells. These signals dock into a receptor protein, initiating a series of cellular and molecular events leading to the rapid up-regulation of bacterial phenotypes often used for surface colonization or association with eukaryote hosts. These phenotypes include biofilm formation (Davies et al., 1998; Lynch et al., 2002), bacterial surface motility (Eberl et al., 1999), production of extracellular products that allow for colonization or attack of hosts (Kievit and Iglewski, 2000), and host-microbe symbioses (Rodelas et al., 1999; Visick and Ruby, 1999).

Although the marine squid–luminescent bacteria symbiosis is one of the model systems for AHL regulation (Visick and Ruby, 1999), the more general role of microbial signaling systems for marine surface dwelling bacteria is not yet well understood. However, it is likely that it will be generally important for bacterial colonization of marine surfaces. Such signaling systems are widespread in bacteria in general (Dunny and Winans, 1999; Winans and Bassler, 2002), including marine species, and the signals are present in situ in both natural aquatic (McLean et al., 1997) and marine (M. Taylor et al., unpublished) biofilms. Both marine (Kjelleberg and Steinberg, 2002) and terrestrial (Bauer and Robinson, 2002) eukaryotes have evolved metabolites that specifically interfere with these signaling systems and thereby inhibit colonization by bacteria. More generally, the potential for chemical communication among marine bacteria at surfaces appears high (Burgess et al., 1999).

Most research on the response of prokaryote colonists to eukaryote hosts has focused on deterrents, and there are numerous studies on the inhibition of microorganisms by extracts or metabolites from marine eukaryotes done in a biomedical or other applied contexts (e.g., Hornsey and Hilde, 1974; Hellio et al., 2001). More recently, deterrence of bacterial colonization has been put in a more ecological context (e.g., Wahl et al., 1994; Maximilien et al., 1998; Kubanek et al., 2002). Interestingly, a number of these later studies highlight specific effects against bacterial colonization per se, rather than generalized bacteriostatic or growth effects. For example, Wahl et al. (1994) demonstrated that metabolites from ascidians

specifically inhibited attachment of bacteria. Halogenated furanones from the red alga *Delisea pulchra* also inhibit specific colonization phenotypes of marine bacteria (Maximilien et al., 1998) at natural concentrations with little or no effects on growth or survival of the bacteria. The likely mechanism for this effect is the inhibition of bacterial signal based regulatory systems by furanones. We (Manefield et al., 1999, 2002; Rasmussen et al., 2000; McDougald et al., 2001) and others (Ren et al., 2001) have shown that furanones specifically interfere with both the AHL and AI-2 systems, resulting in inhibition of phenotypes such as surface motility, exoenzyme production, and biofilm formation and/or stability.

Chemical cues from eukaryotes may also significantly affect the community composition of surface-associated bacterial assemblages. Several chemically rich tropical marine algae have an unusually high proportion of gram-positive bacteria on their surfaces (Jensen and Fenical, 1995), relative to the predominance of gram-negative bacteria in seawater (e.g., Mullins et al., 1995; Cottrell and Kirchman, 2000). Similarly, both culturing studies and pattern analysis of PCR-products via denaturing gradient gel electrophoresis (DGGE) has revealed a high proportion of gram-positive bacteria, particularly high G-C gram-positive strains, on the surface of those portions of *Delisea pulchra* that are richest in furanones (Holmström et al., unpublished). Although key field experiments that directly test the role of eukaryote metabolites on community composition of surface associated bacteria have not yet been done, these and other (Holmström et al., 1996; Burgess et al., 1999; Friederich et al., 2001) studies of the bacterial flora of eukaryotic hosts suggest that such communities differ significantly from those in the surrounding seawater.

A somewhat different, but potentially widespread, mechanism for the inhibition of microbial colonization and infection of marine eukaryotes is the so-called oxidative burst, described for several species of seaweeds (Weinberger and Friedlander, 2000a,b; Bouarab et al., 2001; Kupper et al., 2001). Analogous to the oxidative burst in terrestrial plants, the response is induced by oligosaccharides generated by the degradation (such as through bacterial metabolism) of host cell wall constituents or by bacterial peptides (Weinberger and Friedlander, 2000b). The oligosaccharides induce the production of hydrogen peroxide by the host at concentrations sufficient to kill marine bacteria in laboratory studies. Several seaweeds respond similarly to endophytic algal parasites (Bouarab et al., 2001).

As well as responding to eukaryotic signals, marine prokaryotes—particularly when within microbial biofilms—have a strong influence on the colonization of surfaces by higher organisms (reviewed by Wieczorek and Todd, 1998; Holmström and Kjelleberg, 2000), both in laboratory assays and in the field (Keough and Raimondi, 1995). Most evidence for the effects of biofilms on settlement is for larvae of marine invertebrates (Wieczorek and Todd, 1998), and some larvae (i.e., the tubeworm *Hydroides elegans*) require a biofilm for settlement (reviewed in Hadfield and Paul, 2001). Colonization by at least some macroalgae is also influenced by biofilms (Joint et al., 2000).

Biofilms can enhance or deter settlement of eukaryotic propagules**.** However, the chemical cues from biofilms that are responsible for modulating settlement have mostly not been characterized, or quantified in situ. Inducers of settlement from bacteria investigated to date appear to be polysaccharides or to have polysaccharide components (Kirchman et al., 1982; Szewzyk et al., 1991). Deterrents are diverse. Bacterial ubiquinone inhibits settlement of barnacles (Kon-ya et al., 1994). *Pseudoalteromonas tunicata* produces several distinct metabolites, ranging from a small water-soluble metabolite to a large protein, which individually inhibits bacteria, fungi, algal spores, or invertebrate larvae (reviewed by Holmström and Kjelleberg, 1999). The inhibitory properties of *P. tunicata* are particularly interesting in that its distribution at several sites in both Denmark and near Sydney, Australia, appears restricted to hosts such as *Ulva lactuca* and *Ciona intestinalis*, which lack known endogenous deterrents. [This conclusion of host specificity is based on DGGE and quantitative real-time PCR analysis of bacteria on these and other common potential hosts (Skovhus, unpublished)]. Thus, bacteria such as *P. tunicata* may provide a mechanism for chemical deterrence of colonization for hosts that lack their own defenses (Armstrong et al., 2001; Kjelleberg and Steinberg, 2002).

This later suggestion highlights the potential for cues from living surfaces to emanate from a host or from the associated biofilm (and specific bacteria within the biofilm, e.g., Unabia and Hadfield, 1999). Inducers of settlement from coralline red algae are an excellent example of this issue. Cues associated with coralline algae induce settlement of a range of marine invertebrates, including abalone, seastars, and corals (reviewed by Hadfield and Paul, 2001). In all three of these examples, there is evidence for at least some species that the relevant cues derive from biofilms rather than from the host (Johnson and Sutton, 1994; Roberts, 2001; Negri et al., 2001). Cues acting synergistically between biofilms and hosts may also be a common phenomenon, as Negri et al., (2001) showed for bacterial inducers of settlement of coral larvae, which are effective only in the presence of calcareous substrata. Other difficulties arise because the bacteria that are the putative source of the cues may be unculturable. Finally, although we have argued that bacterial biofilms on the surface of eukaryotes can affect settlement of eukaryote propagules, it is illuminating to consider the biomass of bacteria on a surface and its implications for the amount of cue that can be produced. Typical reported densities from SEM counts (10^6–10^7 cells/cm^2) (Wahl, 1995; Maximilien et al., 1998) of average sized marine bacteria ($1 \times 1 \times 2$ μm) on the surface of marine eukaryotes represent approximately 2–20 μg of biomass per cm^2 of surface (wet weight; assumes 1 $\mu g = 1 \mu m^3$) for all strains combined. At these densities, a typical cue that is active in the low micromolar range would need to represent a significant fraction of the metabolic output of the bacteria to be effective in situ, particularly if exuded.

Methodological Issues: Marine Chemical Ecology and Molecular Microbiology

Understanding the role of microbial biofilms in chemical ecology would be substantially enhanced if we knew what bacteria were out there and in what numbers. It is encouraging that molecular-based tools for community analysis of prokaryotes are developing rapidly (reviewed by Dahllöf, 2002) and are beginning to be applied to assemblages on marine surfaces. The distribution of different bacterial groups can be analyzed directly (microscopically) using fluorescent in situ hybridization (FISH). FISH is limited in a practical sense by the number and taxonomic level of available oligonucleotide probes and by the suitability of different target genes and, thus, is most suitable for targeting specific bacterial species or groups. Other methods for community-wide analyses use extracted DNA directly for slot-blot hybridization or for DNA microarrays in which template genes are probed with DNA or PCR products from the environmental sample. Several other techniques based on pattern analysis of PCR products separated on gels allow for rapid comparisons between different samples or communities. Common examples include denaturing or temperature gradient gel electrophoresis (DGGE and TGGE, respectively). Comparisons of the banding patterns between samples and reference strains in DGGE, or sequencing of individual bands followed by comparisons with sequence libraries, then allow for identification of species. Finally, although in its infancy, detection of bacterial function genes in situ (Dahllöf, 2002), such as those responsible for the production of cues, may ultimately provide an alternative to chemical methods for detecting cues in situ.

EPIPHYTES, PARASITES, SPECIALISTS, GENERALISTS, AND SESSILE VERSUS MOBILE COLONISTS

The consequences of variation in the life history of plants or herbivores have played a fundamental role in the development of theory on plant–herbivore interactions (reviewed by Cronin, 2001). In contrast, the effects of variation in life history or general natural history parameters on chemical mediation of surface-based interactions are largely unexplored. Life history properties of epibiota vary enormously, from bacteria with generation times of hours to invertebrate larvae, which can spend months in the plankton. Mobility of propagules and benthic forms varies from active swimmers to immobility. These differences should in principle provide a rich source of general hypotheses regarding the relative importance of chemical cues for different colonizers. For example, the degree of stringency by which a propagule responds to a cue should reflect both a) the length of time in which it can remain competent and delay settlement and b) the extent to which it or the resulting juvenile can depart the initial site of settlement following a mistake. Thus, we might expect relatively short-lived, nonmotile spores of sessile red algae to be both more

resistant to deterrents and to rely less on specific chemical inducers of settlement than relatively long lived, motile invertebrate larvae (or motile algal propagules). In the only direct test of such ideas, Krug and Zimmer (2000) found no differences in the response of planktotrophic versus lecithotrophic larvae of the slug *Alderia modesta* to carbohydrate cues from its golden green algal host *Vaucheria longicaulis.*

We can also ask whether colonizers that respond strongly to chemical cues are primarily habitat specialists or generalists. Wahl and Mark (1999) have argued that most epibiota are generalists, and many common species or genera of epibiotic algae (e.g., *Enteromorpha, Polysiphonia, Ectocarpus*) or invertebrates (*Hydroides, Bugula*) have wide geographical distributions and occur across a range of habitats or host species. Such organisms might be expected to respond less stringently to specific inducers of colonization than taxa that have a narrow host or habitat range. Some of the strongest responses to inducers are for organisms that are either relatively narrow in their habitat use (Krug and Manzi, 1999; Williamson et al., 2000) or that settle/metamorphose primarily in response to cues associated with conspecifics (Zimmer-Faust and Tamburri, 1994; Tsukamoto et al., 1999).

Several authors have speculated on the role of biofilms in the context of specialized versus generalized cues for colonization. Johnson et al. (1997) suggested that colonization of marine invertebrates was broadly mediated by biofilms, with habitat generalists responding to many components of biofilms in the habitat, and more specialized organisms responding to specific bacterial strains within biofilms. Steinberg et al. (2001) argued that habitat generalists should respond to components of biofilms that are broadly distributed across habitats or hosts, with specialists responding to cues associated with specific hosts or conspecifics. These suggestions raise a number of questions, including: (1) What is the composition of biofilms on different hosts or in different habitats, e.g., is there sufficient variation in biofilm composition across hosts or habitats to provide specialist cues? (2) Are specific strains of bacteria present in sufficient biomass to present a detectable, localized cue for specialist colonizers? Again, the emerging tools of molecular analysis of microbial communities should help answer these questions.

Finally, parasites often represent the most specialized organisms in many systems, yet the chemical ecology of marine host–parasite interactions is largely unexplored. Compounds that deter fungal endophytes or pathogens have been identified (Gil-Turnes et al., 1989; Jensen et al., 1998), but any understanding of how parasites find and infect hosts is largely restricted to a few studies of algal endophytes and their carbohydrate signals. Endophytic algae distinguish between agarophytes (nonhosts) and carrageenophyte hosts (Sanchez et al., 1996) and between different life history stages of host species based on the presence of specific oligosaccharides derived from host plant cell wall (reviewed by Bouarab et al., 2001). Carbohydrates (sulfated galactans) are also the signal for host infection by the fungal parasite *Pythium porphyrae* on its host *Porphyra yezoensis* (Uppalapati

and Fujita, 2000). In this later example, a broad range of red algae induces attachment and encystment of *P. porphyrae*, but a host specific cue is needed to induce the final stage of infection (appressorium formation).

CHEMISTRY, HYDRODYNAMICS, AND DEMOGRAPHY

A major issue for any consideration of chemical cues for colonization is the ability of organisms to detect and respond to relevant cues in the complex hydrodynamic environment found in marine benthic habitats. This topic has been a focus of the field (particularly for invertebrate larvae) for many years (Butman, 1987; Zimmer and Butman, 2000) and is reviewed separately in this volume (Weissburg). However, several relatively recent advances are worth noting. Importantly, cues are now being deployed in the field (e.g., in natural flow regimes) including inducers (Raimondi and Morse, 2000; Browne and Zimmer, 2001) and inhibitors (Henrikson and Pawlik, 1995; Christie et al., 1998; Maximilien et al., 1998; Kubanek et al., 2002). Although various methodological issues remain (e.g., what is the concentration and presentation of cues in artificial matrices relative to the natural source?), these studies have often demonstrated strong effects for chemical cues. The study of Raimondi and Morse (2000) is particularly notable, as they included the cue for coral settlement in a multifactorial field experiment in which the importance of the cue relative to other factors was assessed.

Many propagules of marine organisms are also turning out to be much better swimmers than originally thought, implying that their capacity to respond actively to chemical cues is also much greater than initially believed. Larvae of many fish species are capable of both high speeds and sustained swimming effort (Leis and Carson-Ewart, 1997; Fisher and Bellwood, 2002), and some marine bacteria can swim at hundreds of body lengths per second and take advantage of channels and other microstructures in water as a way of enhancing their chemotactic responses (Blackburn et al., 1998). Other aspects of propagule behavior—such as the extent to which a response to a cue relies on a concentration gradient versus a concentration threshold—can also significantly affect the ability of a propagule to respond to a chemical cue in situ. Ultimately, from an ecological perspective, the goal is to understand how the distribution of a chemical cue in situ affects the demography of the target organism(s) (Raimondi and Morse, 2000; Williamson et al., 2000). The developing focus on in situ analyses and deployment of cues, propagule behavior, and colonization should enhance our understanding of the chemical ecology of colonization in the same way that it has furthered other branches of marine ecology.

CONCLUSIONS

There are good reasons to be positive about the future of research on chemical mediation of colonization in the sea, as the past five years or so have seen a

number of significant advances. These include the first characterizations of many inducers of invertebrate settlement, an explosion in research on bacterial chemical ecology (signaling) and biofilm biology, the first detailed quantifications of surface deterrents, field experiments using purified cues, advances in molecular techniques with considerable utility for the field, and, related to this the increasing interface between molecular mechanisms and ecological consequences (Kjelleberg and Steinberg, 2002; Green et al., 2002). Encouragingly, a more diverse view of the topic appears to be emerging, incorporating novel developments in host–parasite or prokaryote–eukaryote interactions as well as research into longer standing areas such as induction of invertebrate larval settlement.

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MINI REVIEW

ECOLOGICAL CONSEQUENCES OF CHEMICALLY MEDIATED PREY PERCEPTION

MARC J. WEISSBURG,* MATTHEW C. FERNER, DANIEL P. PISUT, and DELBERT L. SMEE

School of Biology
Georgia Institute of Technology
310 Ferst Drive
Atlanta, Georgia, 30332-0230, USA

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Abstract—To locate food, mobile consumers in aquatic habitats perceive and move towards sources of attractive chemicals. There has been much progress in understanding how consumers use chemicals to identify and locate prey despite the elusive identity of odor signals and the complex effects of turbulence on chemical dispersion. This review highlights how integrative studies on behavior, fluid physics, and chemical isolation can be fundamental in elucidating mechanisms that regulate species composition and distribution. We suggest three areas where further research may yield important ecological insights. First, although basic aspects of stimulatory molecules are known, our understanding of how consumers identify prey from a distance remains poor, and the lack of studies examining the influence of distance perception on food preference may result in inaccurate estimation of foraging behavior in the field. Second, the ability of many animals to find prey is greatest in unidirectional, low turbulence flow environments, although recent evidence indicates a trade-off in movement speed versus tracking ability in turbulent conditions. This suggests that predator foraging mode may affect competitive interactions among consumers, and that turbulence provides a hydrodynamic refuge in space or time, leading to particular associations between predator success, prey distributions, and flow. Third, studies have been biased towards examining predator tracking. Current data suggest a variety of mechanisms prey may use to disguise their presence and avoid predation; these mechanisms also may produce associations between prey

* To whom correspondence should be addressed. E-mail: marc.weissburg@biology.gatech.edu

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distributions and flow environments. These examples of how chemical attraction may mediate interactions between consumers and their resources suggest that the ecology of chemically mediated prey perception may be as fundamental to the organization of aquatic communities as the ecology of chemical deterrence.

Key Words—Chemical attraction, chemosensation, community structure, flow, foraging, odor plumes, olfaction, orientation, predator-prey, turbulence.

INTRODUCTION

Over the last 15 years, investigating how natural metabolites mediate biotic interactions has provided valuable insight into the forces shaping the structure of marine communities. Chemical ecology is a vibrant field that helps explain why prey are rare, how animals should allocate energy in their defense, and the role of generalist versus specialist predators in regulating community structure (Hay, 1996). These issues have been studied largely through a focus on chemical defense, and studies of how chemical deterrents affect biotic interactions have become the type of investigation most closely identified with the field of chemical ecology in general. However, just as some chemicals deter consumers, others allow consumers to identify and locate prey from a distance. Although not as fully developed as the study of deterrents, the study of chemical attractants may be of equal importance in determining how biotic interactions affect populations and communities and is an area poised for exciting new developments.

Chemically mediated prey search and identification has been examined in a number of different organisms and in systems ranging from abyssal depths to shallow subtidal and intertidal communities. These studies have emphasized tracking behavior, the properties of sensory systems necessary for odor-guided navigation, or the characterization of attractant compounds. Only rarely have workers investigated the ecological implications of long-distance perception and location of prey in natural communities or realistic laboratory settings. We believe this reflects technical challenges in establishing basic features of chemically mediated attraction, rather than lack of appreciation for, or disinterest in, consequent ecological implications.

The molecular nature of chemical stimuli and the distribution of signals in nature determine how consumers are able to discern and locate prey. Thus, acquiring a basic understanding of the identity of attractant molecules and the physics of odor dispersal is necessary to evaluate the ecological implications of distance perception, but is technically challenging. First, consumers are more likely to use mixtures, perhaps of common compounds, rather than single cues to identify prey. In some cases, the difference between a highly attractive versus a less enticing mixture is simply the ratio of its components, rather than the presence or absence of a distinctive moiety (e.g., Carr and Derby, 1986a,b; Steullet and Derby, 1997).

Identifying attractant stimuli, therefore, is likely to be more difficult than determining the nature of a unique predator deterrent. Second, unlike most deterrent compounds, attractant compounds that mediate distance perception are released into the water. Consequently, extraction and identification of attractants must begin with aqueous solutions derived from intact prey and may be hindered by the considerable dilution of the stimulus. Third, because attractants are transported to animals by flow, it is also necessary to determine the properties of chemical signals in turbulent fluids. However, progress in understanding both the chemical nature of attractant compounds and the role of physical processes in structuring odor signals is now sufficient so that examining ecological implications of chemically mediated predation is feasible.

The purpose of this review is to summarize the progress in understanding chemically mediated prey search and to highlight the potential ecological implications of chemically mediated foraging for community organization. The current state of knowledge enables us only to hint at how prey identification and localization by waterborne cues may affect ecological patterns and processes. Yet, even these brief glimpses may help broaden the field of chemical ecology and move the study of chemical attractance beyond behavioral analysis and characterization of stimulus properties to an understanding of effects on population regulation and community structure. Given the availability of recent reviews on chemical plumes in relation to flow, navigational and guidance strategies, and the chemical nature of attractant compounds (Carr, 1988; Carr et al., 1996; Vickers, 2000; Weissburg, 2000; Zimmer-Faust and Butman, 2000), we focus this review more narrowly and address important issues that are still unsettled or ones where greater understanding may provide insights into basic ecological processes.

THE NATURE OF CHEMICAL ATTRACTRANTS

Despite decades of investigation, the identity of waterborne attractants is still unclear, even for well characterized systems. This surprising fact has two main explanations. First, as noted above, chemical characterization of complex mixtures is technically challenging. Second, the goal of many early studies was to examine physiological properties of the chemosensory system of aquatic animals (e.g., Pearson and Olla, 1977; Carr, 1978; Carr and Derby, 1986a,b) that were poorly known at that time. Consequently, initial bioassays of attractant molecules tended to employ easily identifiable, common, and widely available constituents of animal (or occasionally plant) tissue such as amino acids, nucleotides, and simple sugars or organic acids. Although these chemicals are present in flesh, it is not always clear that they are released in sufficient quantities to mediate distance perception (see below), and they may primarily elicit feeding as opposed to orientation.

There is broad agreement that amino acids and other small molecules are highly stimulatory. Mixtures of 6–25 amino acids, sometimes with quaternary

ammonium compounds, nucleotides, and simple organic acids, have been extensively employed and usually are more effective than any single component (Carr, 1978, 1988; Zimmer-Faust and Case, 1982b; Carr and Derby, 1986a,b; Zimmer-Faust, 1989; Carr et al., 1996), although individual nucleotides can be highly attractive (Zimmer-Faust, 1993). For instance, Carr (1978) used a mixture of betaine and 18 amino acids to assay responses of the shrimp *Palaemonetes pugio*. Still, there is considerable debate regarding the significance of current observations for identfying attractant compounds. Although low-molecular-weight metabolites are clearly important feeding cues, the hypothesized primacy of these substances for orientation has not received a rigorous and comprehensive evaluation (Zimmer-Faust, 1989; Zimmer-Faust and Butman, 2000). Zimmer-Faust (1989) summarizes caveats and provides suggestions on ways to assay attractant compounds that are appropriate for examining distance perception.

Ecologically relevant experiments must present consumers with a flux of attractant molecules similar to that emanating from natural prey. Unrealistically intense chemical cues can stimulate chemosensors that normally mediate near-source search or feeding rather than distance search, because neurons in various appendages may be sensitive to the same suite of compounds but differ in their sensitivity to these chemicals (Caprio et al., 1993; Voigt and Atema, 1992; Weissburg and Derby, 1995). Consumers responding to abnormally elevated signals may not be displaying true distance tracking but, instead, behaviors that occur close to the source or reflexive motions involved in feeding and ingestion. Extracts of ground prey flesh almost certainly result in more intense signals and different mixtures of stimuli than consumers normally experience. Even prey soaks or body washes may produce unnatural stimulus intensities, particularly if prepared using multiple prey soaked in relatively small volumes for long time periods. Few studies have measured flux rates of potential attractant compounds released by living or dead prey as a way to scale flux rates of stimuli in experiments. Limited observations suggest considerable variance in the release of amino acids or ammonia waste products among different prey and differences in the quantity and identity of compounds released by injured, freshly dead, and live prey (Zimmer et al., 1999; Finelli et al., 2000). Mixtures of the 18 most abundant amino acids from bivalve or crustacean flesh released at natural flux rates attracted mud snails (*Ilyanassa obsoleta*) at rates to similar those of fresh carrion, but blue crabs remained disinterested in amino acid plumes mimicking the composition and flux from freshly injured clams (Zimmer et al., 1999; Finelli et al., 2000). Since flux rates in the two experiments differed substantially, the results do not necessarily indicate that crustaceans will not track amino acid plumes, only that injured bivalves do not release these metabolites in sufficient quantities. The concentration of amino acids released by live bivalve prey is less than that found in ambient seawater and below the limit of detection by HPLC, suggesting that amino acids are unlikely to attract consumers to bivalves under natural conditions (Zimmer et al., 1999). The

propensity of marine animals to absorb and not release amino acids (Wright and Manahan, 1989; Manahan, 1990) further suggests they may be generally inappropriate cues. Peptides mediate the predatory response of oyster drills at nanomolar levels (Rittschof, 1990), although the release rates from prey have not been determined. Lobsters appear to be attracted not to amino acids, but to unidentified high molecular weight compound(s) (Zimmer-Faust and Case, 1982a).

The search for a single compound, or even a class of compounds, that stimulates tracking in a particular species may be quixotic, particularly if composition and concentration is as variable across prey species as suggested by current studies (e.g., Zimmer et al., 1999; Finelli et al., 2000). Animals can detect a large number of compounds, and the combinatorial scheme used to identify chemical blends permits the recognition of a variety of mixtures (Derby and Atema, 1988; Sorensen and Caprio, 1997). Given this diversity in production and recognition, why should we expect animals to utilize only a limited range of potential information? It may be that certain compounds make poor signal molecules because they are naturally present at high concentrations with little correlation to prey availability or are not persistent over spatial or temporal scales that would facilitate tracking. A continued focus on identifying metabolites that serve as attractants under natural conditions and the environmental context in which they occur (e.g., flow, turbulence) will be necessary to resolve these issues. Future studies also are necessary to diversify the types of animals for which attractants have been successfully identified. Current data are overwhelmingly derived from predators or scavengers of carrion, with crustaceans being particularly well represented. Few studies have examined the orientation of herbivores to plant extracts or plant-conditioned water (but see Mann et al., 1984; Klinger and Lawrence, 1985; Rahman et al., 2000)

MECHANISMS OF ANIMAL NAVIGATION

Navigation to distant chemical sources often has been investigated in flumes using aqueous extracts of animal tissues (Moore and Atema, 1991; Moore and Grills, 1999) or occasionally, live prey (Weissburg and Zimmer-Faust, 1993, 1994). Perhaps the most important discovery from this work has been that odor signals available to large aquatic animals are affected significantly by turbulence (Zimmer-Faust et al., 1988; Moore and Atema, 1991; Weissburg and Zimmer-Faust, 1993). The resulting signal is complex, unpredictable, and highly variable in space and time (Figure 1), which constrains the strategies animals use to find distant chemical sources. Understanding how interactions between the signal source and environment affect the resultant plume characteristics, and how animals devise effective navigational strategies, can help establish the natural conditions where olfactory-mediated predation will be important.

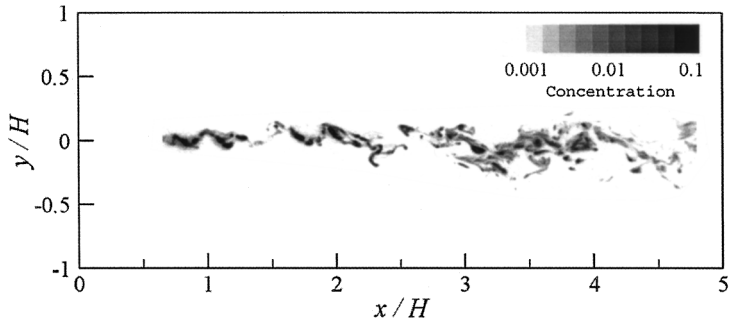


FIG. 1. Chemical concentration field of a turbulent odor plume generated in a laboratory flume. Image shows the chemical concentration field in a chemical plume measured by laser-induced fluorescence. The intensity of light emitted by a dye tracer is captured by a camera and converted to concentration, which is expressed as a proportion of the source concentration (inset). The *y* axis is the cross-stream distance and the *x* axis is the along-stream distance, each expressed relative to the water height, *H* (20 cm). Dye was released from a 3-mm-diam. nozzle located 2.5 cm above the bottom into a flow of 5 cm/sec. Flow is from left to right. Data and further details of this method may be found in Webster et al., (2002).

Crustaceans, fishes, gastropods, and echinoderms all orient to waterborne plumes of stimuli derived from animal flesh (Hamner and Hamner, 1977; Moore and Atema , 1991; Lapointe and Sainte-Marie, 1992; Weissburg and Zimmer-Faust, 1993; Montgomery et al., 1999; Weissburg, 2000). Herbivorous organisms, such as some Echinoidea, are thought to be opportunistic diners that find food through fortuitous random encounters, but this may reflect a lack of realistic tests. Several urchin species move towards palatable seaweeds (Mann et al., 1984; Tertsching, 1989) or selectively to seaweeds that differ in their value as food versus sheltering sites (Hay et al., 1986).

The most useful studies for understanding guidance mechanisms are those that take place in natural environments or in carefully controlled, hydrodynamically appropriate settings generated in flumes. Static tanks or Y mazes cannot accurately reproduce the fluid environment experienced by aquatic foragers and are of limited utility. Flumes have been popular due to the difficulty of visualizing animal behavior and monitoring flow in the field, but such facilities are constrained in the scales of turbulence they can generate. Consequently, we have a poor understanding of how large-scale flow features affect consumer foraging in nature. Limited observations suggest that some animals have difficulty following odor plumes with substantial cross-stream movements produced by large-scale turbulence (Lapointe and Sainte-Marie, 1992).

Crustaceans have been extensively studied in quantified or controlled flow conditions (Moore and Atema, 1991, Moore et al., 2000; Weissburg and

Zimmer-Faust, 1993, 1994; Finelli et al., 2000; Weissburg et al., 2002). Blue crabs, and probably most crustaceans, cannot sample long enough at a given location to estimate local average concentrations reliably due to their relatively rapid movement combined with high variability of turbulent chemical plumes (Webster and Weissburg, 2001; Weissburg et al., 2002). In fact, the difficulty of obtaining accurate mean estimates of many odor signal properties indicates they convey little information when sampled temporally, suggesting that using local averages to gauge upstream progress is unlikely to be successful (Webster and Weissburg, 2001; Weissburg et al., 2002). Blue crabs appear to utilize a combination of odor-triggered response to flow (rheotaxis) combined with spatial sensing of concentration fields (Zimmer-Faust et al., 1995; Webster and Weissburg, 2001; Weissburg et al., 2002). Together these two mechanisms allow animals to move generally upstream towards the source while maintaining contact with the odor plume. Odor-triggered rheotaxis appears common for many animals that search for prey in aquatic habitats (Weissburg, 2000).

Riverine or strongly tidal flows present relatively simple fluid environments where animals may rely commonly on rheotaxis because water movements are nominally unidirectional. Not only is flow information available to help guide navigation, but advection causes a net transport of odor, potentially resulting in a long-distance signal. In these simple flows, animals may follow plumes to baited traps located tens of meters away (Lapointe and Sainte-Marie, 1992; Skajaa et al., 1998), suggesting these regions may be unusually permissive of distant source localization, particularly if current velocities are slow and turbulence minimal (see below). However, animals in wave-driven oscillatory flows (Mead and Koehl, 2000), or in areas where bottom topography may produce complex currents (e.g., reefs) also are proficient foragers. A priori, we expect that the effective tracking distances in these habits will be reduced as compared to behavior in unidirectional flows, due both to the difference in physical transport and the absence of a reliable flow cue. Oscillatory flows limit the net movement of odor, whereas complex flows may create a more difficult signal to track because of both large-scale meander and the negative impact of small-scale turbulence (see below).

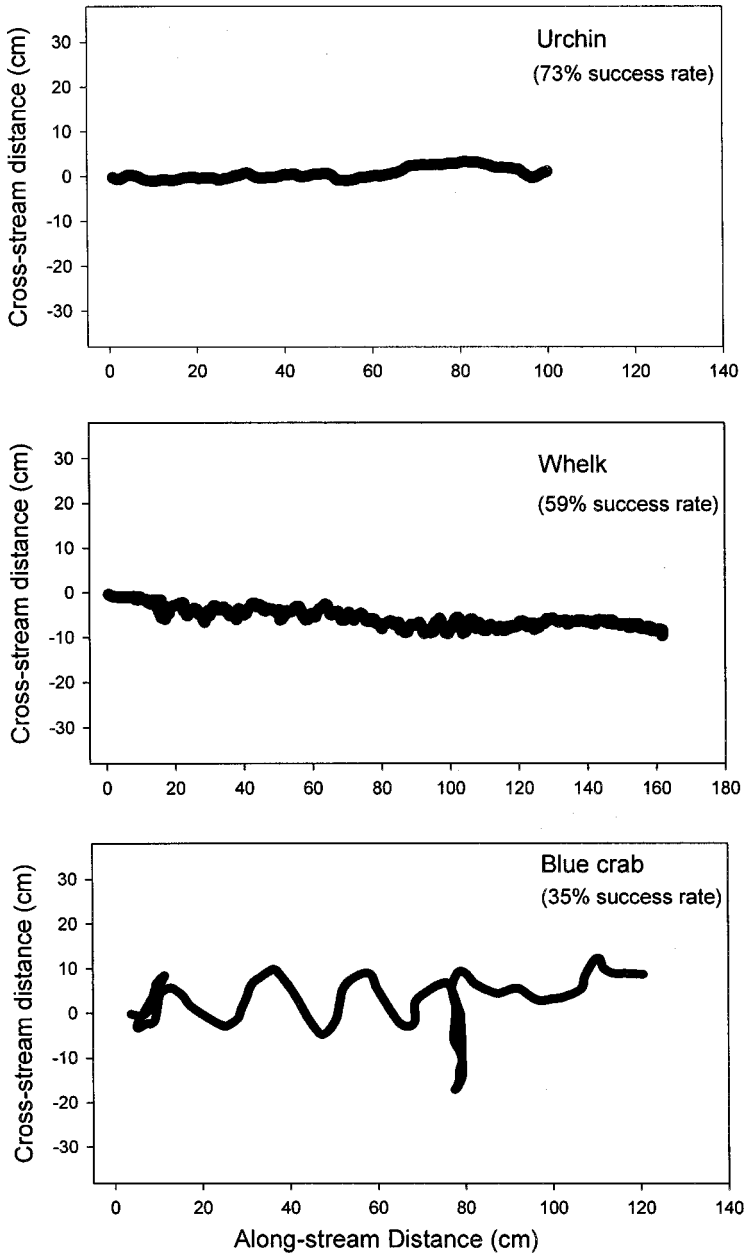
Animals inhabiting more complex flow environments may reduce their reliance on rheotaxis. Lobsters are thought to use predominantly chemical information during tracking, although firm tests are lacking and conclusions have been based on indirect evidence from studies in limited flow conditions or from behavior of animals with experimentally induced sensory deficits (Reeder and Ache, 1980; Devine and Atema, 1982; Moore and Atema, 1991). These observations provide evidence regarding the importance of chemical information but cannot exclude a rheotactic component. More direct tests are rare, but would include probing for upstream locomotion in response to brief chemical pulses or water velocities below which navigation is impaired (Weissburg and Zimmer-Faust, 1993; Mafra-Neto

and Cardé, 1994, 1998; Finelli et al., 2000), or examining whether animals follow flow streamlines. Flow provides potentially valuable information that could greatly greatly enhance the efficiency of navigation, making it important to establish the conditions under which animals employ rheotaxis.

Animals may commonly use accessory cues such as flow or gravity to supplement information from chemical signals. An odor source that generates substantial turbulence in its wake or releases odor in the form of a vertical jet creates a plume that is initially elevated above the substrate and attaches to the bottom downstream of the source. Thus, consumers of substrate-bound food may interpret a sudden loss of signal to mean that they are close to the source. Foragers sometimes use the substrate as an accessory cue (thigmotaxis) and concentrate search efforts on the bottom following signal loss. Even capable swimmers may fail to find sources above the bottom when employing this strategy (Tamburri and Barry, 1999). Similarly, some pelagic animals tend to follow odors downward once they have detected a chemical signal (Hamner and Hamner, 1977), which might limit their ability to track mobile prey as opposed to passively sinking objects.

Turbulence disrupts odor plumes, renders individual filaments less detectable, and increases larger scale plume meander (Murlis, 1986; Moore et al., 1994; Weissburg et al., 2002). As expected, blue crabs show diminished search success and efficiency when challenged with more turbulent flows (Weissburg and Zimmer-Faust, 1993). Crayfish rarely seem inconvenienced by these conditions, and in fact, may search more effectively in moderate turbulence (Moore and Grills, 1999). The sensory basis for these behavioral differences in response to elevated turbulence remains unknown. For some animals, detrimental effects of turbulence may be a function of the relationship between animal sensory capabilities and length and time scales of odor features (Weissburg, 2000). Animals that move quickly through a plume perceive odor on a very coarse scale and have little choice but to respond to intense, instantaneous plume features. Sluggish predators may have the opportunity to sample more finely in time or space, resulting in an ability to estimate the local plume concentration that would be less affected by increased turbulence. Indeed, initial studies with whelks and echinoderms suggest that they maintain largely similar performance as turbulence is increased and have little apparent difficulty tracking odor in conditions where blue crabs are inefficient (Figure 2). In addition, blue crab search success is significantly lower at flow speeds

FIG. 2. Comparison of prey tracking in various aquatic animals. The figure shows typical paths of urchins (*Lytechinus variegatus*), whelks (*Busycon canaliculatum*), and blue crabs (*Callinectes sapidus*) tracking odor plumes in a laboratory flume. The odor source consisted of metabolites released from freshly dead, intact bivalves. All other conditions are the same as described in Figure 1. Shear velocity is approximately 3.1 mm/sec. Search success is given in parentheses.


Urchin
(73% success rate)
Whelk
(59% success rate)
Blue crab
(35% success rate)
Cross-stream distance (cm)
Along-stream Distance (cm)

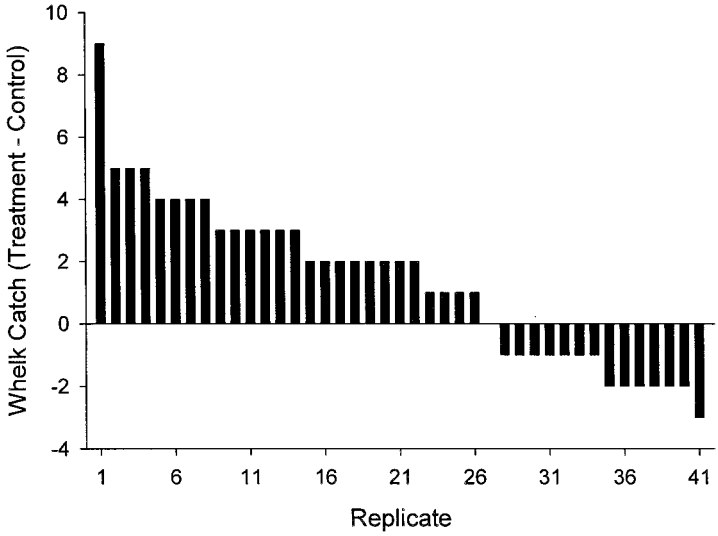


FIG. 3. Response of whelks to elevated turbulence in the field. Data show the difference between the numbers of whelks caught in traps modified to increase turbulence versus unmodified (control) traps. Traps generating increased turbulence caught an average of 1.4 more whelks than control traps and a pair-wise t-test indicates a significant effect of trap type on whelk catch ($t = 3.34$, $P < 0.001$, $N = 41$).

of 5 cm/sec than 1 cm/sec (Weissburg and Zimmer-Faust, 1996), whereas neither whelks (M. Ferner and M. Weissburg, unpublished data) nor urchins (D. Pisut and M. Weissburg, unpublished data) show decreased success across these speeds. In an initial study (M. Ferner and M. Weissburg, unpublished data), whelks were more attracted to baited traps that were modified to enhance turbulence when pairs of modified and unmodified traps were placed in similar flow environments (i.e., on opposite stream banks; Figure 3).

ECOLOGICAL IMPLICATIONS

Many aquatic organisms that hunt via olfaction can exert large effects on prey species, and sometimes, entire communities. Digging predators such as crustaceans or gastropods often control bivalve abundance (Virnstein, 1977; Micheli, 1997; Nakaoka, 2000), and their predatory activities cause local disturbances resulting in the death or emigration of non-target organisms (Pennings and Bertness, 2001). Such disruptions create considerable environmental heterogeneity that may be at least partially responsible for community patchiness. Urchins may aggregate in response to food, and dense urchin assemblages can denude large tracts of

vegetation (e.g., Estes et al., 1998; Rose et al., 1999). Given the current knowledge of chemically mediated foraging, it is opportune, perhaps essential, that we take steps to examine the ecological consequences of this process. Below we highlight a few avenues that may be particularly well suited in this regard.

Prey: Cueless or Clueless?

Until now, we have been fascinated by predator behavior, while prey have been relegated to the role of convenient stimulus sources. This short-sightedness may be understandable, but ignores the fact that predators evoke responses even from prey species often considered as having limited behavioral flexibility. As examples, when gastropods sense foraging crabs, they increase shell thickness and decrease foraging (Trussel, 1996). Mussels respond to consumers by thickening shells, increasing byssal attachment strength, and forming denser aggregations (Cote and Jelnikar, 1999; Leonard et al., 1999). Although prey cannot necessarily eliminate the expulsion of metabolites used as signals by predators, they may reduce the apparency of these emissions to make tracking more difficult for predators.

Given that some predators best track prey in conditions of smooth flow, regions of intense turbulence may represent a hydrodynamic refuge (Weissburg and Zimmer-Faust, 1993). This refuge may be spatial, resulting in the dispersal of prey into habitats with turbulent flow. It also may be temporal if prey actively liberate chemical cues during periods of high flow to render their signal undetectable. Some animals cannot feed unless they also expel waste plumes (e.g., bivalves). Prey attempting to hide their chemical signature consequently may suffer reduced growth as a nonlethal effect of minimizing their apparency to consumers. In fact, bivalves grow more slowly in the presence of predators, even when shielded from direct interactions (Nakaoka, 2000), suggesting alteration in the duration, and perhaps distribution, of feeding times.

Prey may have other means of disguising their presence beyond altering the timing of chemical release. Given the tendency of animals to search the ground for odor sources, animals may make themselves more difficult to locate if they expel plumes with sufficient vertical momentum so that chemical cues are transported far downstream before reattaching to the substrate. This strategy would be particularly effective when animals are in slow flows, since under these conditions the jet will travel farther from the substrate before its vertical momentum is lost (Moore et al., 1994). Animals that can capitalize on the ability to produce vertical jets may enjoy an advantage in slower flows relative to other potential prey.

Appropriate interindividual spacing also may reduce the risk of detection. Close neighbors may produce signals that interact (Monismith et al., 1990), increasing the cross-stream span of the plume as well as the flux of potential stimulants. Predators, therefore, may be more easily alerted to the presence of prey and find the signal easier to track than when they encounter plumes from a single individual.

Larger interindividual spacing may be particularly beneficial for prey that have a minimal impact on the surrounding flow (e.g., burrowing bivalves). Animals whose bodies generate substantial flow disturbances might actually benefit from aggregating more closely to enhance turbulence. If prey generate sufficient turbulence, their plume may disperse so quickly that it becomes difficult to detect even when prey density is high. These interactions may have implications for patterns of demography across regions of different odor-tracking permissiveness that have yet to be tested.

Significantly, prey often may use waterborne odors from predators to modify their behavior in response to immediate threats (Kats and Dill, 1998; Trussel, 1996). Habitats favorable for odor tracking may, therefore, increase both the ability to forage and to detect predators. Many animals, such as echinoderms, gastropods, or small crustaceans, are simultaneously both predators and prey. The ultimate consequences of enhanced odor detection are not predictable a priori but present a number of intriguing possibilities. For instance, foragers with more acute sensing of potential predators may be favored even in habitats where they may be more vulnerable to detection, whereas those less able to perceive predator odors may be restricted to more hydrodynamically marginal environments.

Competition and the Race for Food

The mounting evidence that olfactory foragers may differ in their skill at tracking signals in turbulent conditions suggests that individual predator species may be tuned for efficient foraging in given environments and that turbulence is a relevant dimension of the environment affecting the distribution and abundance of potential competitors. Even in the absence of strong competition, animals may gravitate towards conditions where they experience the greatest foraging success, suggesting that habitat choice, and consequently, the intensity of predation by particular species, may be affected by turbulence levels.

The partitioning of olfactory environments along a turbulence intensity axis may be particularly important for slow versus rapidly moving consumers, as these represent the end points of a spectrum of potential navigational strategies. In areas of low turbulence, a rapidly moving forager has the advantage, since plume conditions allow an animal responding to momentary bursts of intense odor to locate the source with speed and precision. A consumer employing this strategy reaches its goal before an animal using a time-consuming strategy dependent on local averaging. In more turbulent flows, the race for food is not necessarily to the swift, since local averaging may be the only effective tracking strategy where odor fields are dilute and bursts of high concentration are rare. As a result of the suitability of different navigational strategies across flow regimes, slow-moving time-averagers may be proficient at predation or scavenging in turbulent habitats, whereas more rapid crablike strategies should predominate where turbulence is

low. This hypothesis has yet to be examined. Since turbulence is affected by factors acting on regional (e.g., tidal amplitude) or local (e.g., bottom topography) scales (Wright, 1989; Mann and Lazier, 1991), the interaction between hydrodynamic environments and sensory strategies may produce differences in patterns of predation both between and within systems.

Predators that use different signal structures to locate prey would be expected not only to have distinct habitat preferences and predatory efficiencies even in similar environments, but perhaps preferences for different prey characteristics. By example, slow-moving consumers capable of computing local concentration can integrate in time to estimate flux, which for a given current speed is proportional to the product of metabolite concentration and release rate. Temporal integration is not necessary for rapidly moving animals that use perception of intense odor bursts to navigate. Flux is essentially meaningless for these consumers; instantaneous estimates of burst concentration provide the information used to find prey. Interestingly, crabs and whelks vary in size selectivity for their bivalve victims, with whelks tending to consume larger prey (Nakaoka, 2000). A number of potential explanations exist, but one possibility is that crabs and whelks do not use the same signal properties to guide navigation and, therefore, respond differently to prey of different sizes. Assuming larger prey have higher release rates, as opposed to releasing more concentrated stimuli, flux will increase, whereas properties of individual odor bursts may change little, if at all. Larger bivalve prey might, therefore, present a more attractive signal for whelks than smaller prey, which would be consistent with gastropods such as *Ilyanassa* that demonstrate increased aggregation to odor sources with greater fluxes (Zimmer et al., 1999). Crabs simply may be unable to discern prey size from a distance, and upon contact reject larger items in favor of smaller bivalves, which they can open more easily (Arnold, 1984). Alternatively, crabs may use some unknown odor or hydrodynamic feature, such as excurrent jet size or velocity, to select smaller prey. Chemically mediated strategies capable of producing differences in predation might serve to define, at least partially, functional guilds. Mobile aquatic predators often have been assigned to guilds based on foraging mode [e.g., burrowing, digging, subsurface (Woodin, 1983) or stomach contents (Hines et al., 1990)]. These criteria may not be completely diagnostic when predators utilize different plume features in order to find their prey. Patterns of community regulation may be contingent on the degree to which its members are functionally redundant (members of the same guild; Strong, 1992), making accurate guild delineation necessary to thoroughly understand community dynamics.

Patterns of Preference

Many consumers in aquatic systems eat a variety of living prey (e.g., Hines et al., 1990), and some also may derive considerable nutrition from scavenging

(Britton and Morton, 1994). Factors that mediate patterns of prey choice have not been explored, particularly in realistic field settings. In the field, diet will be determined by the ability to detect an item from a distance, and once the prey has been contacted, by its palatability, degree of chemical or morphological defense, and nutritive value. In contrast, many feeding assays are conducted in small arenas where distance detection is minimized, so that postdiscovery factors are the primary arbiter of feeding decisions. This may confound accurate identification of consumer preference and result in unrealistic estimates of consumer impact on particular diet items. Nutritious, tasty, but undetectable food may be less prevalent in consumer diets than suggested by assays failing to account for distance perception.

Diet preferences also might be contingent on whether predators use differences in the quality (composition) of the odor signal or merely the quantity of stimulatory molecules, to identify and track their prey. The difference between these two scenarios is significant, since dining choices mediated by odorant composition reflect specialization on particular prey items, and specialists may exert different effects in communities than generalists. Understanding the basis of diet preferences, therefore, may help illuminate the role of given predators in aquatic communities. There is abundant evidence that aquatic predators posses the capability to identify chemical mixtures characteristic of particular food items and discriminate among them (Carr and Derby, 1986; Derby and Atema, 1988; Wight et al., 1990; Sorensen and Caprio, 1997). As noted above, it is unclear whether these stimulus mixtures are relevant to the task of source localization, as opposed to ingestion behavior. Chemical composition of amino acid solutions derived from injured prey has no significant impact on attractiveness of the snail *Ilyanassa* when presented at equal flux rates (Zimmer et al., 1999). One possibility is that scavengers use general and ubiquitous cues such as amino acids or nucleotides, whereas animals seeking live prey use more specific molecules. This supposition is consistent with the little evidence available regarding attractant compounds for scavengers and predators. Although *llyanassa* is primarily a deposit feeder, it must scavenge carrion for survival and reproduction (Curtis and Hurd, 1985). Juveniles of the predatory snail *Urosalpinx* feed exclusively on balanoid barnacles, which they find using a specific peptide cue (Rittschof, 1990).

CONCLUSIONS

The chemical ecology of deterrent compounds has provided insight into species interactions by successfully blending techniques from analytical chemistry, behavior, and ecology. The study of attractant compounds is poised to make important contributions, but in order to do so, must adopt a similar interdisciplinary perspective. The basic tools of chemical analysis, fluid dynamics, and behavior are now sufficiently advanced and, in some cases, have been combined in novel ways to examine odor tracking. The continuation of these efforts, particularly realistic

field experiments and in situ manipulations of chemical and fluid environments, has the potential to address many intriguing issues. With increased attention and effort, the ecology of attraction may become as important to understanding marine communities as the study of chemical deterrence.

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MINI REVIEW

CHEMICAL ECOLOGY OF MARINE MICROBIAL DEFENSE

SEBASTIAN ENGEL, PAUL R. JENSEN, and WILLIAM FENICAL*

Center for Marine Biotechnology and Biomedicine
Scripps Institution of Oceanography
University of California, San Diego
La Jolla, California 92093-0204, USA

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Abstract—Because marine animals and plants are continuously exposed to a large diversity of potentially harmful microorganisms, it seems reasonable to hypothesize that potential hosts might produce bioactive compounds to deter microbial attack. However, little is known about how host metabolites may defend against harmful microbes or facilitate the growth or colonization of helpful symbionts. While there is a large body of literature describing the antimicrobial activities of marine secondary metabolites, we are only now beginning to understand how these compounds function in an ecological context. For example, there is mounting evidence that nontoxic concentrations of secondary metabolites can have significant effects on microbial behavior, suggesting that certain host–microbe interactions are chemically mediated. Herein, we discuss the importance of employing ecologically relevant assays to elucidate microbiological effects and the need to develop a better understanding of host–microbe associations within an ecologically realistic context. Continued research in this field along with improved techniques will certainly provide further insight into how microbes have influenced the evolution of secondary metabolite production in marine organisms.

Key Words—Antimicrobial defense, secondary metabolites, microbial attachment, microbial challenges, microbial interactions, microbial swarming.

* To whom correspondence should be addressed. E-mail: wfenical@ucsd.edu

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INTRODUCTION

The interdisciplinary field of chemical ecology involves investigations to understand the ecological function and evolutionary significance of secondary metabolite production by animals, plants, and microbes. In general, secondary metabolites are thought to enhance the fitness of the producing species, a concept supported by many studies that have documented their roles in mediating intra- and interspecific interactions (recently reviewed by Rosenthal and Berenbaum, 1992; Harborne, 2001). In the ocean, predator–prey interactions, spatial competition, and larval settlement cues have been the central focus of marine chemical ecology (e.g., Hay and Fenical, 1988; Paul, 1992; Pawlik, 1992; Hay, 1996; McClintock and Baker, 2001). Studies in this field have led to a solid understanding of how secondary metabolites from marine animals and plants affect other species and support the now well-accepted theory that secondary metabolites provide important defensive functions against consumers and competitors. Despite this progress, the effects of secondary metabolites on potentially harmful marine microorganisms remain largely unknown. This is somewhat surprising, considering that marine pathogens can have devastating effects on entire populations (Litter and Litter, 1995; Correa and Sanchez, 1996; Smith et al., 1996; Martin et al., 2002), suggesting that marine microorganisms exert significant selective pressures on marine animals and plants. Investigating the microbiological effects of secondary metabolites in an ecological context is extremely important if we are to develop a clear understanding of the extent to which microorganisms have influenced the evolution of secondary metabolites.

There is a strong ecological rationale for the maintenance of antimicrobial chemical defenses by marine invertebrates and plants. Seawater, unlike air, typically contains 10^7 viruses, 10^6 bacteria, 10^3 fungi, and 10^3 microalgae/ml (Reinheimer, 1992), all of which include members that have been identified as causative agents in a variety of marine infectious diseases (Andrews, 1976, 1979; Sparks, 1985a,b; Correa, 1997). Given that marine invertebrates and plants lack cell-based immune responses and are continuously exposed to a broad array of potentially deleterious microorganisms, it is reasonable to hypothesize that the production of bioactive secondary metabolites could act as a fundamental mechanism of antimicrobial defense. While secondary metabolites have previously been suggested to function as defenses against marine pathogens (Paul, 1992; Hay, 1996), methods to address this hypothesis are only now being developed and, as a result, we are now getting our first glimpse of their potential roles in mediating interactions between the producing organisms and microbial enemies.

Further rationale for investigating the role of secondary metabolites in mediating microbial interactions comes from the well-documented observations that marine plants and animal are host to a variety of often specific microorganisms (Sieburth, 1975, 1979). Contrary to inanimate surfaces, which are colonized in a

rapid and predictable manner by a diverse assemblage of marine microbes (ZoBell and Allen, 1935; Wahl, 1989), biotic surfaces frequently harbor species-specific microbial communities (Baker and Orr, 1986; Wardell et al., 1983; Wahl, 1995) that can be highly variable and distinct from those found in the surrounding environment. While little is known about the potential role of secondary metabolites in the regulation of these associations, an understanding of the types of microbial behaviors that may be chemically mediated, such as chemotaxis, adhesion, swarming, and biofilm formation (recently reviewed by Lawrence et al., 1995; Fletcher, 1996; Steinberg et al., 2001; Amsler and Iken, 2001), can provide a framework within which to investigate the ecological relevance of molecules that mediate microbial physiology and behavior.

The purpose of this paper is to explore to what extent secondary metabolites from marine organisms influence the behavior of potentially pathogenic, or otherwise harmful, marine microorganisms. Herein, we provide a brief review of how crude marine extracts and purified secondary metabolites produce ecologically relevant effects on marine microbes. Although several assays have been developed to investigate the effects of secondary metabolites on the behavior of harmful microbes (reviewed by Jenkins et al., 1998), the difficulty of developing bioassays that are relevant to natural systems has clearly been a major limitation to the development of this field. Despite these challenges, there now exists a small but compelling body of literature that provides strong evidence for the role of secondary metabolites in antimicrobial chemical defense.

CHEMICAL INTERACTIONS BETWEEN MARINE ORGANISMS AND ASSOCIATED MICROBES

The significant interest in the pharmacological potential of marine natural products has resulted in a wealth of literature describing antimicrobial activities of marine secondary metabolites against biomedically important microorganisms (Rinehart et al., 1981; Reicheldt and Borowitzka, 1984; Carté, 1996). In addition, studies investigating the ecological role of antibiotics have reported antimicrobial effects of secondary metabolites against marine bacteria (Azumi et al., 1990; Kim, 1994; Koh, 1997; Newbold et al., 1999). While these studies provide evidence that marine secondary metabolites can have antimicrobial properties, the ecological significance of these activities is uncertain. In fact, several studies have noted a lack of correlation between antibiotic activity and epibacterial abundance (Bakus et al., 1985; Davis et al., 1989; Wahl et al., 1994) suggesting that either antibacterials are not involved in the regulation of surface-associated bacteria or that traditional antibacterial bioassays may not be adequate predictors of the ecological functions of secondary metabolites.

The detection of antimicrobial activity measures the direct inhibition of microbial growth, which is only one potential mechanism by which epibacterial

abundance and pathogenicity can be controlled. Aside from toxicity or growth inhibition, marine secondary metabolites may also influence microbial surface colonization by inducing a negative chemotactic response (Chet et al., 1975; Chet and Mitchell, 1976; Amsler and Iken, 2001) or by affecting various stages of the attachment and colonization process (Steinberg et al., 2001; Kjelleberg and Steinberg, 2002). Assays that measure the effects of extracts or purified secondary metabolites on microbial behavior (reviewed by Jenkins et al., 1998), such as chemotaxis, adhesion, and surface swarming, have the potential to demonstrate the extent to which microbial surface colonization is chemically mediated. Based on available data, behavioral assays seem inherently more predictive of ecological function than assays that simply measure toxicity. Although only a handful of studies have employed behavioral assays to investigate the ecological roles of secondary metabolites, these have been used effectively to relate the activity of organic extracts and isolated compounds from marine invertebrates (Wahl et al., 1994; Slattery et al., 1995; Bryan et al. 1996) and plants (Jensen et al., 1998; Maximilien et al., 1998) to epibacterial abundances on the producing organisms. In the following, a brief review of these studies is provided in an effort to explore the extent to which secondary metabolites from marine organisms mediate interactions with cooccurring and potentially deleterious microbes.

Animal–Microbe Interactions

Wahl et al. (1994) were among the first to provide evidence that marine organisms chemically mediate bacterial surface colonization. In their study, epibacterial abundances on two cooccurring ascidians, *Polyclinum planum* and *Cystodytes lobatus*, were investigated. Scanning electron micrographs revealed that the surface of *P. planum* was heavily colonized by diatoms and a variety of bacteria, while that of *C. lobatus* was virtually free of associated microorganisms. The numbers of culturable bacteria from both ascidian species corroborated the SEM results and led the authors to explore the hypothesis that epibacterial abundances were chemically controlled in these two temperate ascidian species.

To test this hypothesis, Wahl et al. (1994) investigated the effects of ascidian extracts on microbial attachment and growth. The ability of bacteria to attach to a surface was tested using a bioassay in which bacteria isolated from both ascidians were allowed to settle onto agar surfaces containing extract-treated and control areas. The effects of ascidian extracts on bacterial settlement were determined by comparing the numbers of cells attaching to extract treated and control surfaces. In addition, the extracts were tested for antibacterial activity using standard disk diffusion assays (Acar, 1980) against 36 bacterial strains isolated from the surfaces of both ascidians. While extracts from both ascidian species exhibited variable and generally low levels of antimicrobial activity, the extracts of the sparsely colonized

C. lobatus, tested at concentrations below those estimated to occur in the tissues of this species, were found to significantly inhibit bacterial attachment relative to controls. In contrast, the numbers of bacteria settling on agar surfaces containing extracts of the heavily colonized *P. planum* were significantly greater than those settling on controls. These results provided good evidence that the settlement of surface-associated microorganisms was chemically controlled in both ascidian species. Interestingly, settlement activities of ascidian extracts correlated well with epibacterial abundance, while antimicrobial activities did not, providing some of the first experimental evidence that antimicrobial chemical defenses may function by mechanisms other than toxicity.

Slattery et al. (1995) used a similar assay to investigate the effects of extracts from three Antarctic soft corals (*Alcyonium paessleri*, *Clavularia frankliniana*, and *Gersemia antarctica*) on the settlement and growth of cooccurring bacteria. While extracts of all three soft corals exhibited minimal antimicrobial activity, chloroform extracts from *Alcyonium paessleri* and *Gersemia antarctica*, as well as methanol extracts from *Gersemia antarctica*, significantly inhibited bacterial attachment in a modified slide assay (Little et al., 1986; Wahl et al., 1994). This provided further evidence that extracts tested at nontoxic concentrations can have significant effects on bacterial behavior, leading the authors to conclude that both *A. paessleri* and *G. antarctica* rely on secondary metabolites to inhibit bacterial attachment to their surfaces (Slattery et al., 1995).

Ecologically relevant effects on cooccurring marine bacteria have also been demonstrated with echinoderm body wall extracts. While potent antimicrobial activities have been reported for crude extracts and specific compounds (saponins) isolated from echinoderm species (Rinehart et al., 1981; Bryan et al., 1994), Bryan et al. (1996) were the first to investigate the effects of echinoderm extracts on bacterial settlement. Using a spectrophotometric 96-well microtiter plate assay, modified from Shea and Williamson (1990), Bryan et al. (1996) assessed the effects of echinoderm body wall extracts on the attachment of the marine bacteria *Deleya marina* and *Alteromonas luteo-violacea*. Testing extracts at natural tissue concentrations, the authors demonstrated that the extracts of three echinoderm species significantly inhibited bacterial attachment, while the extracts of eight species significantly enhanced attachment. Interestingly, the extract of one of the three species that inhibited attachment (*Goniaster tesselatus*) was shown in a previous study (Bryan et al., 1994) not to be toxic to microbes.

Additional evidence that secondary metabolites from marine animals may be involved in antimicrobial chemical defense comes from a study of Caribbean sponges. A survey conducted by Newbold et al. (1999) revealed that crude organic sponge extracts have a wide range of antimicrobial activities against ecologically relevant marine bacteria. To further investigate the chemical basis of these activities, Kubanek et al. (2002) used a settlement assay modified from that of Jensen et al. (1998) to guide the isolation of the active constituents in the extracts.

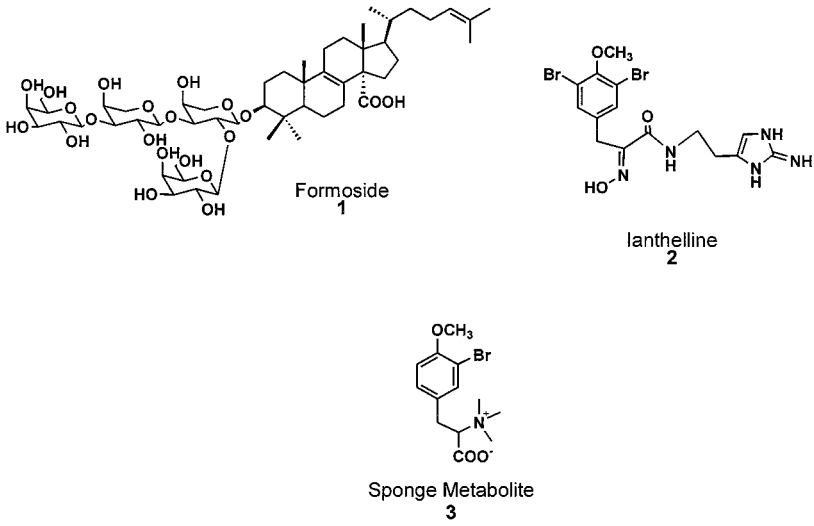
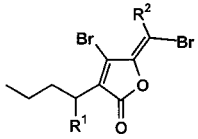


FIG. 1. Animal-derived secondary metabolites with potent behavioral effects on cooccurring marine bacteria.

Using the marine bacterium *Vibrio harveyi* as a guide, Kubanek et al. (2002) demonstrated that formoside (**1**, Figure 1), isolated from the sponge *Erylus formosus*, significantly inhibited bacterial attachment at nearly two orders of magnitude below natural tissue level concentrations. Using the same assay, Kelly et al. (unpublished.) demonstrated that ianthellin (**2**, Figure 1), isolated from the sponge *Ailochroia crassa*, and the bromotyrosine derivative **3** (Figure 1), produced significant inhibitory effects on bacterial attachment and swarming, respectively. Thus, it was possible to assign the activities associated with crude extracts to individual secondary metabolites and to demonstrate that these compounds had significant effects on bacterial behavior at whole tissue concentrations. However, it is important to note that whole-tissue concentrations might not necessarily be the same as surface concentrations, which cooccurring microbes are more likely to experience in nature. With the exception of Kubanek et al. (2002), none of the previously discussed studies determined surface concentrations of extracts tested. Conducting settlement assays with surface concentrations rather than whole tissue concentrations could provide valuable information to interpret results in a more ecologically relevant way.

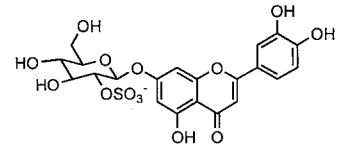
Plant–Microbe Interactions

Although few marine plants have been investigated for their ability to mediate microbial interactions through the production of secondary metabolites, the



4a, R^1 = H, R^2 = Br
4b, R^1 = H, R^2 = H
4c, R^1 = OAc, R^2 = H
4d, R^1 = OH, R^2 = H

Furanones



Luteolin 7-O-β-D-glucopyranosyl-2"-sulfate
5

FIG. 2. Plant-derived secondary metabolites with potent behavioral effects on cooccurring marine bacteria.

results obtained to date have been intriguing. The most in depth studies have been performed on the red alga, *Delisea pulchra*, which is known to produce a series of structurally related halogenated furanones (**4**, Figure 2) (Kazlauskas et al., 1977; de Nys et al., 1993). Scanning electron micrographs of the alga revealed that epibacterial abundances near the holdfast were significantly higher than those found on the apical tips (Steinberg et al., 1997), where the highest concentrations of halogenated furanones were detected (de Nys et al., 1996). These observations led Maximilien et al. (1998) to explore the hypothesis that halogenated furanones are involved in mediating bacterial colonization on the surface of *D. pulchra*.

To test this hypothesis, Maximilien et al. (1998) measured epibacterial abundances and distributions on *D. pulchra* as well as on three cooccurring algal species and tested the effects of crude extracts and halogenated furanones in a series of microbial behavioral assays. To ensure that the bacteria used in these assays were exposed to ecologically relevant furanone concentrations, Maximilien et al. (1998) conducted their assays within a range of concentrations found to occur on the surface of *D. pulchra* (de Nys et al., 1998). Naturally occurring surface concentrations were determined by dipping the plant into nonpolar solvents for 40 secs, a period determined not to damage surface cells (de Nys et al., 1998). Overall, Maximilien et al. (1998) determined that crude extracts of tissues from *D. pulchra* had no significant inhibitory effect on the growth of 144 ecologically relevant bacterial strains isolated from the surface of *D. pulchra* and the surrounding environment. Since toxicity did not provide an adequate explanation for the low numbers of bacteria observed on the surface of *D. pulchra* relative to other cooccurring algae, Maximilien et al. (1998) proceeded to test the effects of crude extracts and purified furanones on the ability of bacteria to swim, attach, grow, and swarm, four important characteristics associated with surface colonization. These experiments included some of the first field experiments addressing the microbiological effects of marine secondary metabolites and represent an important step towards testing extracts and purified compounds against an ecologically relevant, natural assemblage of microorganisms.

Initial field studies revealed that crude extracts and furanones **4b** and **4c** (Figure 2) strongly inhibited the ability of bacteria to attach to a surface (Maximilien et al., 1998). These results led to further studies aimed at determining whether host versus nonhost strains of bacteria differed in their ability to swim, attach, grow, and swarm in the presence of these compounds. To perform these experiments, three bacterial strains isolated from the surface of *D. pulchra* and three isolated from the surfaces of nearby rocks were selected. Although the effects on the four behavioral characteristics varied among isolates, a trend in the results indicated that attachment was inhibited in nonhost bacteria while growth and swarming were inhibited in host bacteria (Maximilien et al., 1998). These results may explain why nonhost strains were not found on the alga's surface and why host strains had limited surface distributions. It is interesting to note that secondary metabolites may control the density of surface associated microbes, allowing growth of a limited number of epibiotic microbes rather than maintaining an axenic surface. This strategy might represent one mechanism by which the host minimizes the risk of counter adaptation, which would be likely to occur if secondary metabolites would be toxic to associated microbes.

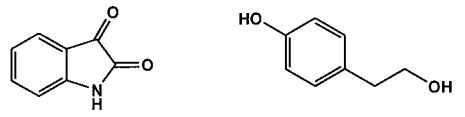
Other evidence that plant secondary metabolites affect cooccurring microorganisms comes from the study of a group of protist decomposers known as thraustochytrids. These fungilike microorganisms occur widely in coastal marine environments (Moss, 1986; Raghukumar et al., 1992) and are known to be important decomposers of plant material (Miller and Jones, 1983; Bremer, 1995). It has long been suspected that phenolic compounds produced by marine plants function as a chemical defense (Sieburth and Conover, 1965; Sieburth, 1968) by inhibiting decomposers such as thraustochytrids from attacking live plant material. However, there has been little chemical evidence implicating specific compounds in this process. Working with extracts of the the seagrass *Thalassia testudinum*, Jensen et al. (1998) isolated and described a sulfated flavonoid (**5**, Figure 2) with potent physiological and behavioral effects on the co-occurring thraustochytrid, *Schizochytrium aggregatum*. Initial experiments revealed that significantly fewer thraustochytrids were associated with healthy living tissue of *T. testudinum* than with tissue that had been sterilized and placed back into the field for 48 hr. Using *S. aggregatum* as a guide, the possibility of a chemical basis for this observation was investigated and led to the isolation and characterization of the water soluble flavonoid luteolin 7-*O*-β -D-glucopyranosyl-2″-sulfate (**5**, Figure 2). This compound inhibited the growth of *S. aggregatum* with an IC_{50} of 270 μg/ml, a concentration about 15 times lower than the average values in seagrass tissues. This compound was also found to significantly inhibit the attachment of *S. aggregatum* in an assay modified from Wahl et al. (1994), supporting the author's contention that it may function as a defense against marine decomposers (Jensen et al., 1998). Although sulfated flavone glycosides had been previously isolated from terrestrial plants, they are most prevalent in halophytes and their production was believed to represent a

physiological adaptation to the marine environment (Harborne, 1977). This study provided the first evidence that a specific compound belonging to this class of secondary metabolites may function in antimicrobial chemical defense.

Current work in our laboratory has also indicated that other marine algae produce secondary metabolites that inhibit the development of known marine pathogens and potentially deleterious microorganisms. Recently, J. Kubanek et al. (personal communication) found that extracts of the tropical brown alga *Lobophora variegata* inhibited the growth of four of six potentially pathogenic microbes. One of these strains, *Dendryphiella salina*, an ascomycete known for its role in the decomposition of algal tissue, was chosen for the bioassay-guided chemical fractionation of the *L. variegata* crude extract. These assays were performed in microtiter plates and the extracts, fractions, or pure compounds were incorporated into agar substrates at whole tissue concentrations and tested for their effects in fungal growth. This assay led to the isolation of a molecule that possesses extremely potent antifungal activities not only against *D. salina* but also against the known algal pathogens *Lindra thalassia* and *Lulworthia fucicola.* In a separate study, M. Puglisi et al. (personal communication) investigated the effects of extracts of the green alga, *Penicillus captiatus*, on the ubiquitous marine pathogen *L. thalassiae*. Using a similar microtiter plate assay, two unusual secondary metabolites were isolated (not yet fully identified) with potent antifungal activity. These studies clearly demonstrate that marine plants produce secondary metabolites that, when tested at whole tissue concentrations, have significant effects on marine plant pathogens and saprophytes.

Microbe–Microbe Interactions

In an interesting twist to the concept of antimicrobial chemical defense, there is evidence that symbiotic microbes can chemically defend a host against microbial infection. This phenomenon was demonstrated in a series of experiments probing the observed resistance of crustacean eggs toward a fungal pathogen (Gil-Turnes et al., 1989; Gil-Turnes and Fenical, 1992). Crustacean egg masses are primarily composed of proteins and polysaccharides (Hunt, 1966; Flower et al., 1969), making them excellent substrates to support microbial growth. Scanning electron micrographs of the surfaces of eggs from the estuarine shrimp *Palaemon macrodactylus* revealed that they were colonized by a monoculture of a bacterium that was subsequently identified as an *Alteromonas* sp. (Gil-Turnes et al., 1989). When the bacteria were removed by treatment with penicillin, the egg mass became extremely susceptible to infection by the oomycete *Lagenidium callinectes*, a common crustacean pathogen (Armstrong et al., 1976; Fisher et al., 1978). Colonization by *L. callinectes* caused death of embryos within hours. Reintroduction of the cultured bacterial symbiont to the penicillin-treated eggs restored resistance against *L. callinectes*, providing strong evidence that the epibiotic bacteria provide



2,3-indolinedione
"Isatin"
6

4-hydroxyphenethyl alcohol
"Tyrosol"
7

FIG. 3. Bacterial metabolites with potent antifungal activities.

a chemical defense for the host. Chemical investigation of the cultured bacterium led to the isolation and description of 2,3-indolenedione (isatin) (**6**, Figure 3), which was found to be a potent inhibitor of *L. callinectes* growth (Gil-Turnes et al., 1989). A similar study revealed that the egg masses of the American lobster, *Homarus americanus*, were colonized by a gram-negative bacterium that produced the antifungal metabolite 4-hydroxyphenethyl alcohol (tyrosol) (**7**, Figure 3) in pure culture (Gil-Turnes and Fenical, 1992). The production of antimicrobial chemical defenses by symbiotic microorganisms represents an added level of complexity to the mechanisms by which marine plants and animals avoid the deleterious effects of microbial pathogens.

SUMMARY

It is becoming increasingly clear that marine secondary metabolites can have significant ecological effects on potentially deleterious microorganisms. The effects on bacteria and fungi documented to date, which range from antibiotic activities to specific effects on microbial behavior, suggest that in some marine animals and plants, secondary metabolites function as an antimicrobial chemical defense. These defenses may be straightforward, e.g., the direct inhibition of a pathogen via the production of an antibiotic (Jensen et al., 1998), or they may be more subtle, e.g., the induction of a negative chemotactic response or the interference with a chemically mediated signaling pathway required for bacterial surface colonization (Givskov et al., 1996; Kjelleberg et al., 1997). What has become evident from this work is that when careful studies are performed using ecologically relevant microorganisms and realistic estimations of secondary metabolite concentrations, the complex effects of these adaptive metabolites can be deciphered.

Although the evidence that marine invertebrates and plants maintain antimicrobial chemical defenses is growing, a number of obstacles must be overcome before a complete understanding of the ecological importance of these microbiological effects can be obtained. Among the most pressing issues is the estimation of the concentrations of secondary metabolites experienced by microorganisms in

nature. Testing extracts and purified compounds at natural or whole-tissue concentrations is a logical starting point and has proven effective in studies of predator deterrence; however, these methods may not translate to the microbial level. Methods to estimate surface concentrations, such as that developed by de Nys et al. (1998), represent important innovations in this regard; however, this task becomes more challenging in the case of spatially complex organisms like sponges, and they do not account for compounds that are released directly into the surrounding seawater. Equally important is choosing the appropriate microorganisms against which to test the hypothesis of chemical defense. Test microorganism selection remains a significant challenge, considering how little is known about which microorganisms are potentially deleterious. A logical place to start is with known pathogens or cooccurring microorganisms that possess the capacity to colonize a surface. It is also critical that assay results, such as antibacterial activities, are correlated with microbiological observations, such as bacterial abundance, and that field assays be developed so secondary metabolites displaying activities in the laboratory can be tested against natural assemblages of microorganisms. Although progress is being made in all of these areas, it is clear that continued advances in this field will require methodological innovations as well as a better understanding of the nature of microbial interactions with marine invertebrates and plants.

Another important conclusion that can be drawn from the studies performed to date is that assays that measure toxicity alone are inadequate predictors of antimicrobial chemical defenses. There is mounting evidence that secondary metabolites can have significant effects on microorganisms at nontoxic concentrations (Wahl et al., 1994; Slattery et al., 1995; Bryan, 1996; Jensen et al., 1998; Maximilien et al., 1998), and for this reason, assays that measure behavioral responses such as chemotaxis and the ability to swim, attach, and swarm, are proving to be more informative in assessing the spectrum of possible ecological effects than assays that simply measure toxicity. Results from behavioral assays have also made it possible to demonstrate that secondary metabolites can attract microorganisms (Wahl et al., 1994; Bryan, 1996) and, thus, these metabolites may help ensure colonization by preferred epibionts. Studies addressing the roles of secondary metabolites as chemical attractants will help elucidate the potential roles of secondary metabolites in maintaining favorable microbial associations and symbioses.

While it is clear that marine invertebrate and plant secondary metabolites can have significant effects on microorganisms, the effect of pathogenic and saprophytic microbes on the host are not well understood. Secondary metabolites that affect microbial processes such as swimming, attachment, growth, and swarming, have been implicated as having defensive functions for the producing organism. However, by definition, a defensive function implies that in the absence of the chemical defense, the producing organism would sustain harm from the microbe. Thus, a clear understanding of the microorganisms in the ocean, and their associations with plants and animals is of fundamental importance to any conclusions

drawn from experiments investigating the microbiological functions of secondary metabolites.

The compelling conclusion that plants and animals appear to maintain antimicrobial chemical defenses leads to subsequent questions about the degree to which diverse selective pressures, such as predation and microbial infection, have influenced the evolution of secondary metabolite biosynthesis. It remains unclear if different compounds function in different defensive roles or if individual compounds have multiple ecological functions. If the latter is correct, then the evolution of antimicrobial chemical defenses in response to microbial challenge may be constrained due to other ecological pressures (Schmidt et al., 1995) thereby compromising the host's ability to respond to microbial attack. This may explain the occurrence of microbial epidemics such as infections by *Aspergillus sydowii* in Caribbean gorgonians (Smith et al., 1996) and by bacteria in coralline algae (Litter and Litter, 1995). Since the roles of plant and invertebrate metabolites in deterring macroscopic predators are relatively well known, it will be important to take these results into consideration when assessing the evolutionary significance of antimicrobial chemical defenses.

Overall, there is a growing body of evidence supporting the hypothesis that marine plants and animals maintain antimicrobial chemical defenses. With methodological improvements and a better understanding of host-microbe interactions, it should be possible to provide further insight into the adaptive strategies against microbial pathogens and establish the extent to which secondary metabolites regulate microbial interactions.

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MINI REVIEW

CHEMICAL DEFENSE OF EARLY LIFE STAGES OF BENTHIC MARINE INVERTEBRATES

NIELS LINDQUIST*

Marine Sciences Program
University of North Carolina at Chapel Hill
Institute of Marine Sciences
Morehead City, North Carolina 28557, USA

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Abstract—Accurate knowledge of factors affecting the survival of early life stages of marine invertebrates is critically important for understanding their population dynamics and the evolution of their diverse reproductive and life-history characteristics. Chemical defense is an important determinant of survival for adult stages of many sessile benthic invertebrates, yet relatively little consideration has been given to chemical defenses at the early life stages. This review examines the taxonomic breadth of early life-stage chemical defense in relation to various life-history and reproductive characteristics, as well as possible constraints on the expression of chemical defense at certain life stages. Data on the localization of defensive secondary metabolites in larvae and the fitness-related consequences of consuming even a small amount of toxic secondary metabolites underpin proposals regarding the potential for Müllerian and Batesian mimicry to occur among marine larvae. The involvement of microbial symbionts in the chemical defense of early life stages illustrates its complexity for some species. As our knowledge of chemical defenses in early life stages grows, we will be able to more rigorously examine connections among phylogeny, chemical defenses, and the evolution of reproductive and life-history characteristics among marine invertebrates.

Key Words—Chemical defense, juveniles, larvae, life-history evolution, marine invertebrates, mimicry, predation, reproductive characteristics, symbiosis, taxonomic constraints.

* E-mail: nlindquist@unc.edu

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INTRODUCTION

The risk of early life stage mortality is a critical component of models and debates concerning the evolution of the different reproductive and life-history characteristics exhibited by marine invertebrates (e.g., Vance, 1973; McEdward, 1997). In these discussions, predation often is assumed to be a major source of mortality for early life stages. The purpose of this review is to bring forward ideas and hypotheses regarding the role of chemical defenses against predation in the ecology of early life stages (i.e., eggs to juveniles) of marine invertebrates and the potential involvement of secondary metabolites in the evolution of marine invertebrate life histories and reproductive characteristics.

Many marine organisms from low to high latitudes and in both benthic and pelagic habitats are effectively defended from diverse types of predators by noxious secondary metabolites (see reviews by Paul, 1992; Pawlik, 1993; Hay, 1996; McClintock and Baker, 2001). Defensive chemistry is particularly common among soft-bodied sessile invertebrates such as sponges, octocorals, and ascidians that can dominate some subtidal habitats where rates of herbivory and predation are especially intense (e.g., coral reefs). Given the demonstrated importance of chemical defenses for the survival of adult marine organisms and for multiple life stages of terrestrial plants (Janzen, 1971; Swihart and Bryant, 2001), it seems reasonable that they may also be important throughout the ontogeny of marine species. Chemical defense of marine larvae is becoming increasingly well documented, and because McClintock et al. (2001) recently reviewed this topic, only a brief synopsis of the primary literature on larval chemical defense is presented. Instead, I place the chemical defenses of early life stages (egg, embryos, larvae, and juveniles) in a larger evolutionary framework and ask how these defenses may affect the evolution of, for example, egg and larval size, brooding versus broadcast spawning, timing of spawning, and dispersal potential. Additionally, some less obvious, yet potentially important, ecological and evolutionary implications for the chemical defense of early life stages are discussed.

OVERVIEW OF EARLY LIFE-STAGE CHEMICAL DEFENSE

Direct examination of defensive secondary metabolites in eggs and larvae has been conducted for saponins in sea stars (Howden et al., 1975), didemnin cyclic peptides and tambjamine alkaloids in tunicates (Lindquist and Fenical, 1991; Lindquist et al., 1992), terpenes in soft corals and gorgonians (Coll et al., 1995; Harvell et al., 1996; Slattery et al., 1999), and halogenated phenols and alkanes in polychaetes (Cowart et al., 2000) (Figure 1). Additional studies on the palatability of early life stages or their extracts illustrate the breadth, and potentially limits, of chemical defenses among taxonomically diverse marine invertebrates. Eggs,

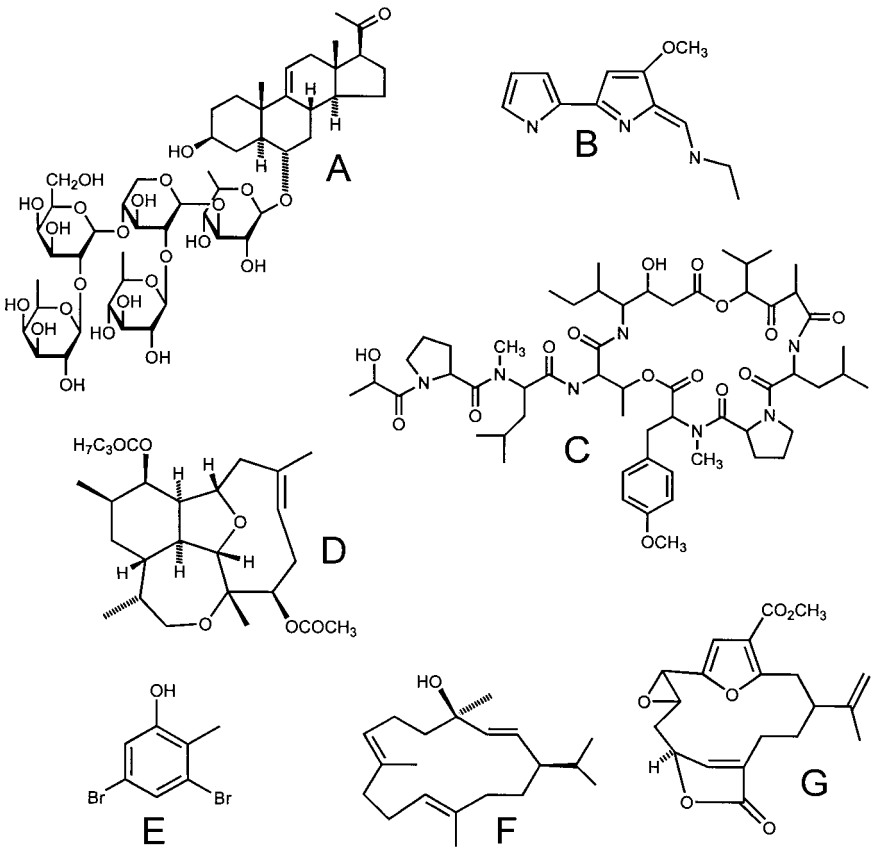


FIG. 1. Representative structures of secondary metabolites isolated from eggs, embryos, and larvae of marine invertebrates: (A) saponin, *Acanthaster planci*; (B) tambjamine E, *Sigillina signifera*; (C) didemnin B, *Tridemnin solidum*; (D) asbestinin, *Briareum asbestinum*; (E) bromophenol, *Capitella* sp; (F) (−)-*epi*-thunbergol, *Lobophytum crassum*; (G) pukalide, *Sinularia polydactyla.*

embryos, and larvae most commonly reported to be distasteful and to have deterrent extracts are from sessile invertebrates like sponges (Lindquist, 1996; Lindquist and Hay, 1996; Uriz et al., 1996; McClintock and Baker, 1997), brooding gorgonians and soft corals (Harvell et al., 1996; Lindquist and Hay, 1996; Kelman et al., 1998), hydroids (Lindquist and Hay, 1996), bryozoans (Lindquist and Hay, 1996; Lopanik et al., unpublished), and colonial tunicates (Young and Bingham, 1987, Lindquist et al., 1992). The few mobile taxa with species reported to have unpalatable eggs and larvae include sea stars and holothurian (sea cucumbers) (Lucas et al., 1979; Cowden et al., 1984; McClintock and Vernon, 1990; McClintock and

Baker, 1997; Iyengar and Harvell, 2001) and shell-less gastrods (Pawlik, 1993), but also a crustacean (Luckenbach and Orth, 1990) and a polychaete (Martin et al., 2000). In contrast to the above findings, Bullard et al. (1999) using taxonomically diverse field-captured larvae found no evidence of chemical defense among crustaceans, gastropods, and polychaetes—three very specious taxa that dominate the meroplankton (see also Cowden et al., 1984). Bullard et al. (1999) did find some suggestion of chemical defenses among the few sponge, cnidarian, nemertean (a "worm" unrelated to polychaetes), and sea star larvae caught.

TAXONOMIC CONSTRAINTS AND EVOLUTIONARY IMPLICATIONS

Orians and Janzen (1974) asked, "Why are embryos so tasty?", after pointing out that birds, reptiles, amphibians, and fishes all lose large proportions of their eggs and larvae to predators and that the evolution of distasteful eggs and larvae should be favored. They hypothesized that eggs and larvae generally lack chemical defenses due to: (1) autotoxicity, especially for actively growing tissues, (2) energetic constraints, and (3) trade-offs between deterrence and potential developmental rates of the young. Given the increasing number of marine species now known to have chemically defended eggs and larvae (McClintock et al., 2001), the lack of distasteful eggs and larvae noted by Orians and Janzen (1974) is likely due to the rarity of chemical defenses among vertebrates in general. Taxonomic constraints on the production of chemical defenses may exist among marine invertebrates as well.

The most chemically rich invertebrate taxa in the oceans are generally sessile, soft bodied, and clonal. Their soft tissues and inability to move away from predators make them highly vulnerable, but the noxious and toxic chemicals they possess enhance their persistence in hostile habitats where predation is intense. Clonal growth likely facilitates the evolution of chemical defenses because distasteful individuals can survive bouts of partial predation, while promoting learned aversion by co-occurring predators. In contrast, solitary organisms are less likely to recover from a significant loss of tissue. This also is true of marine larvae, even those of clonal invertebrates, because they are small solitary organisms.

Marine invertebrates have diverse reproductive and larval traits that offer differing degrees of protection against potential predators (Young and Chia, 1987; Morgan, 1995). The timing of spawning, either seasonal or daily, can help early life stages avoid some types of predators. The small size of many larvae, as well as being nearly transparent or cryptically colored, helps them avoid detection by visually foraging predators. Smaller larval size typically correlates with higher adult fecundity, but smaller larvae must usually feed (= planktotrophy) and grow in the plankton for weeks to months before reaching a size that is competent to settle. This may lead to extensive dispersal on currents and an increased risk of never

encountering suitable adult habitat. Some larvae are highly mobile and can often escape when attacked. Others produce an array of potential morphological barriers, such as spines and setae. Morphological defenses may deter some predators (Morgan, 1995), while other consumers easily circumvent morphological barriers (Cowden et al., 1984; Bullard et al., 1999). If larval development is partially or entirely benthic, developing embryos and larvae are usually dispersed within gelatinous egg masses or enclosed in tough protective capsules. Many of the above traits co-occur among larvae of taxa that are not known to be chemically defended and may be their best bets for reducing larval losses to predators.

In contrast to certain morphological traits, chemical defenses can be broadly effective against diverse types of predators (Paul, 1992; McClintock and Baker, 2001), and some simultaneously diminish competition, fouling, disease, or stress from UV radiation (e.g., Schmitt et al., 1995; Stachowicz and Lindquist, 1997). While palatable larvae appear to be selected to avoid exposure to, or detection by, predators (e.g., by being small, translucent, released at night, and dispersing away from the benthos), chemically defended larvae are commonly conspicuous, day active, and remain near the benthos during their short swimming stage. Because many chemically defended larvae are unharmed following attacks by fish and benthic particle feeders (Young and Bingham, 1987; Lindquist, 1996; Lindquist and Hay, 1996), the seascape "seen" by chemically defended larvae may be one largely devoid of successful predators, whereas predators abound in the same habitat as "seen" by a poorly defended larva. The high survival of distasteful larvae following attack and rejection by predators also indicates that the evolution of chemical defenses could occur at the level of an individual larva—evoking group or kin selection to explain the evolution of larval chemical defenses is not necessary. Jackson (1986) pointed out that philopatry (i.e., settling near one's parents) appears common among clonal benthic invertebrates and can result in dense, genetically closed populations (Grosberg and Quinn, 1986); thus, kin selection could further facilitate the evolution of chemical defenses in early life stages and adults.

If the "problem" of predation has largely been solved by the production of chemical defenses, then the larval stage should be free to evolve traits that reduce losses due to other sources of mortality, even at subsequent life stages. As newly settled juveniles, the survival of benthic invertebrates is enhanced by increased size (e.g., Jackson, 1985). Because large larvae typically produce large juveniles, selection could strongly favor increased larval size, if larger size is not constrained by opposing disadvantages. Selection for larger size may occur with chemically defended larvae because the corresponding increase in apparency to visual predators should not increase a larva's risk of mortality and may actually enhance predator recognition and learned avoidance of distasteful larvae. Because distasteful larvae are at less risk from visual predators, they can also be released during daylight hours when they can use strong photic cues to detect protected settlement sites (dark

cracks or crevices) where early juvenile stages will experience less stress from high UV or indiscriminate scraping by large fishes and invertebrates (Young and Chia, 1984; Lasker et al., 1998; Maldonado and Uriz, 1998). Consistent with the above hypotheses, chemically defended larvae are commonly large and released during daylight hours (Lindquist et al., 1992, 1997; Lindquist and Hay, 1996), whereas predators generally constrain palatable larvae from exhibiting such traits.

Rigorous examination of the roles chemistry plays in the life-history evolution of marine invertebrates is both helped and hampered by various constraints. The lack of diverse secondary metabolism among some taxa allows for broad comparisons at the level of phyla, but confounding factors are numerous. For example, sponges are the most primitive metazoans and have only a rudimentary differentiation of their cells and tissues (Ruppert and Barnes, 1994). Thus, sponges probably could not evolve a highly differentiated larva with elaborate feeding capabilities. Despite this drawback, comparing the chemical and life-history characteristics of sponges with colonial tunicates, which are primitive chordates, is enlightening. Tunicates have great cellular diversity and tissue differentiation and are certainly capable of having evolved a feeding larval stage [see hypotheses regarding the origins of the pelagic tunicates called larvaceans (Ruppert and Barnes, 1994)]. Yet ecologically, sponges and colonial tunicates are equals in many respects. Both groups are sessile, benthic filter feeders known to possess a diverse array of biologically active secondary metabolites (Faulkner, 2002, and previous reviews cited therein). Many species in both taxa brood large, aposematically colored larvae that are spawned during daylight hours and that are chemically defended against predators (Young and Bingham, 1987; Lindquist et al., 1992; Lindquist and Hay, 1996). Thus, it seems plausible to hypothesize that their ability to produce bioactive compounds at various life stages has played a role in the convergence of their life histories. Opportunities to investigate within-phyla or within-class relationships between chemistry and life histories could also focus on the Echinodermata and Ascidiacea, respectively, both of which exhibit reproductive diversity among chemically rich and chemically depauperate sub-taxa.

CHANGES IN CHEMICAL DEFENSES AMONG LIFE STAGES

Identifying changes in defensive chemistry among different life stages of marine invertebrates may provide clues about chemically mediated interactions important to the survival of large adults on exposed benthic substrates, small larvae in planktonic environments, and small juvenile stages that typically inhabit physical or biological refuges from large mobile predators. Will one compound adequately defend against different suites of predators in vastly different habitats, or has selection tailored the chemistry at each life stage to act against those predators most capable of inflicting mortality at each life stage?

The first comparison of secondary metabolites between adult and larval stages was for the crown-of-thorns sea star (Howden et al., 1975). In both eggs and adults, they found similar levels of the same saponins; a pioneering study subsequently demonstrated that these saponins deterred feeding by planktivorous reef fishes (Lucas et al., 1979). Later investigations found the same class of secondary chemistry among the different life stages of colonial tunicates (Lindquist et al., 1992), gorgonians (Harvell et al., 1996), soft corals (Slattery et al., 1999), and polychaetes (Cowart et al., 2000), although concentrations of individual metabolites can vary widely among different life stages. Secondary metabolites found in all life stages are possibly broadly effective at deterring diverse guilds of predators, but this hypothesis has not been tested.

In contrast, some marine invertebrates show a distinct lack of chemistry in one or more life stages, a situation potentially useful for testing the hypotheses that these metabolites: (1) only deter predators most likely to prey on the chemically rich life stage, (2) have costs that outweigh the benefits at some life stages, and (3) are possibly unrelated to defense against consumers. Coll et al. (1995) provide examples of egg-specific terpenes in broadcast-spawning soft corals that seem unrelated to predator deterrence (but see Slattery et al., 1999). These terpenes do, however, attract sperm, thereby increasing fertilization rates. Harvell et al. (1996) reported fluctuations in levels of asbestinin diterpenes among life stages of the surface-brooding gorgonian *Briareum asbestinum*, with dips in their concentrations occurring at the embryo–larval and larval–juvenile developmental transitions. They suggest that these dips conform to the hypothesis of Orians and Janzen (1974) by reducing chemical levels when tissues are metabolically very active. While virtually nothing is know about the origins of defensive chemistry in early life stages, the fluctuations measured by Harvell and coworkers in *B. asbestinum* suggest active diterpene synthesis at all life stages.

Early post-settlement stages of many sessile marine invertebrates are morphologically vulnerable to predation (Gosselin and Qian, 1997; Hunt and Scheibling, 1997), yet the cost of a chemical defense may outweigh the benefits for newly settled juveniles. High rates of growth should be of overwhelming importance for the early post-settlement stages because the probability of individual mortality decreases with increasing size (Jackson, 1985). If this hypothesis is generally true, and assuming a trade-off exists between growth and defense, will the production of a chemical defense found in the adult and their reproductive products (i.e., eggs, embryos and larvae) always shut down during the early juvenile stages to increase rate of growth? If so, at what age or size does production of the chemical defense re-emerge? Growth at the expense of defense should not be favored, if predation on undefended juveniles is intense, and rapid growth does not provide a sufficient escape from predators. Selective settlement into cryptic habitats (cracks and crevices), which is common for larvae of many sessile benthic invertebrates, exposes newly settled juveniles to small predatory mesofauna, such as polychaetes,

gastropods, and crustaceans, that can be abundant within refuges (up to $\sim 58,000$ individuals/m^2 on coral reefs) (Klumpp et al., 1988). Thus, do predatory mesofauna within refuges represent a significant source of early post-settlement mortality that can be intense enough to select for defense over growth?

Osman and Whitlatch (1995) provide examples of the dramatic impact mesoconsumers can have on populations of early-stage juveniles. They found that small gastropods could eliminate new recruits of two species of colonial ascidians, whereas juveniles of a third, morphologically similar ascidian were largely unpalatable, suggesting a chemical defense. For the two species with palatable juveniles, their susceptibility to predation by snails dramatically decreased 2 weeks after settlement. In addition to possibly attaining a refuge in size, the reduction in predation could be due to an upswing in the production of chemical defenses by the 2-week-old juveniles. Unfortunately, simultaneous assessments of growth rates and the palatability of juvenile extracts have not been reported for these three ascidians.

Uriz et al. (1996) report palatability data for adults, larvae, and juveniles of two Mediterranean sponges that suggest chemical defenses operate to defend both species from predation, but not at all life stages. Their use of different predators in assays with adult versus larval and juvenile stages precludes a direct comparisons among all three life stages. However, among their larvae, 1-week-old juveniles, and 2-week-old juveniles, all larval and juveniles stages of *Dysidea avara* were rejected by a co-occurring fish, whereas only the 2-week-old juveniles of *Crambe crambe* were rejected. These results suggest that there may be considerable variation in palatability among larval and early juvenile stages.

LOCALIZATION OF CHEMISTRY

Two important questions regarding potential ecological roles for bioactive secondary metabolites in larvae, as well as the other life stages, are, "Where is the chemistry localized?" and "Can it be mobilized?". Compounds that are internalized and immobile are unlikely to function in larval defense as the cue that predators detect and respond to in rejecting a larva. This hypothesis flows from observations that the surface of distasteful larvae attacked and rejected by fish are not disrupted and that most attacked and rejected larvae survive, settle, and successfully metamorphose (Young and Bingham, 1987; Lindquist and Hay, 1996). Lindquist and Hay (1995) demonstrated that some predators do not detect and respond to the presence of physiologically harmful secondary metabolites but (1) if capable of learned aversion, quickly generalize on the "flavor" of the food and use cues other than the toxic metabolites to reject the prey, or (2) if incapable of learned aversion, suffer chronic depressions in fitness that could select for individuals among predator species that recognize and reject small toxic prey as food. These results

indicate that harmful secondary metabolites sequestered within larvae could play a role in defense but one which would require some larval mortality before predators learn or evolve to recognize and avoid chemically harmful early life stages.

To date, few studies provide any data on the localization of ecologically important metabolites in early life stages of marine invertebrates. Lindquist and Fenical (1991) suggested that the yellow-colored tambjamine alkaloids that defend larvae and adults of the tropical tunicate *Atapozoa* sp. (= *Sigillina signifera*) from predatory fishes are localized in the animal's highly colored granular amebocyte blood cells. These blood cells circulate throughout the larvae, and many occur near the larval surface. It is not known whether these compounds are exuded by either the larvae or the adults. Although it is not readily apparent how compound localization will be routinely determined for the small and difficult-to-work-with early life stages, innovative methodologies that address this issue will significantly advance our understanding of larval and juvenile chemical ecology.

MIMICRY

For invertebrate species that typically co-occur, have overlapping reproductive seasons, and produce chemically defended larvae (e.g., sponges on coral reefs), selection could favor the evolution of Müllerian mimicry complexes in which unpalatable larvae of different species converge on a similar aposematic color pattern. Although Dunlap and Pawlik (1996) found no correlation between the color of adult sponges and their palatability, aposematic coloration seems to be a common characteristic of many distasteful larvae (Young and Bingham, 1987; Harvell et al., 1996; Lindquist and Hay, 1996), even coming from adults lacking bright coloration (N. Lindquist, personal observation). Tests to document Müllerian mimicry complexes among chemically defended larvae have not been conducted.

The existence of Batesian color mimics of unpalatable, aposematically colored larvae also lacks empirical support. Presently, no larvae are reported to be both palatable to fishes and brightly colored. However, Young and Bingham (1987) demonstrated that learning among visually orienting predators could select for Batesian color mimics. They found that fish reject palatable tunicate larvae dyed to match the bright orange color of unpalatable larvae from the tunicate *Ecteinascidia turbinata* after previously sampling and rejecting *E. turbinata* larvae.

A novel Batesian mimicry system suggested by current data, but which is highly speculative, is that predators reject larvae of some species, not because the larvae possess noxious or toxic metabolites, but simply because they "taste" like a chemically defended larva. Although this hypothesis sounds paradoxical, it is based on observations of distasteful larvae surviving attacks unscathed, which strongly suggest that fish and other predators taste or smell surface-associated chemicals that cause them to reject a larva as prey. Thus, if toxic larval chemistry on the

surface can not be detected (Lindquist and Hay, 1995) or is internalized, larvae of a few chemically defended species potentially could reduce predation rates on non-chemically defended species simply if, on the surface, the chemically benign larvae mimic the flavor or odor of unpalatable larvae. This taste-based mimicry could act independently of visual cues, implying that even nonvisual predators, such as benthic particle feeders, might evolve to reject chemically benign larvae. Empirical studies with birds (e.g., Roper and Marples, 1997) and mammals (e.g., Provenza et al., 2000) and modeling studies (Augner and Bernays, 1998) indicate that flavors and odors can act as cues for toxic foods, and, thus, support mimicry systems.

DEFENSE BY SYMBIOSIS

A provocative hypothesis, originating primarily among marine natural products chemists (e.g., Kobayashi and Ishibashi, 1993; Faulkner et al., 1999), is that microbial associates are the true source of biologically active compounds isolated from some species of chemically rich invertebrates, primarily among sponges, bryozoans, and colonial tunicates. Many of these invertebrates harbor microbial symbionts and possess secondary metabolites with structural similarities to known microbial metabolites, yet few studies have convincingly demonstrated symbiont production of these metabolites. Two of these studies (Gil-Turnes et al., 1989; Gil-Turnes and Fenical, 1992), however, showed that externally brooded embryos of some crustaceans harbor surface-associated bacteria shown to produce secondary metabolites that inhibit infection by pathogenic microorganisms. Because crustaceans are not generally known to possess bioactive secondary metabolites, these studies illustrate the power of bacterial symbionts in protecting early life stages against pathogens, which is potentially a common phenomenon among many invertebrate taxa (see also Benkendorff et al., 2000).

More recently, Lindquist and coworkers (unpublished data) have found another example of a symbiont contributing to host defense, including its early life stages. These are unusual symbioses on Indo-Pacific coral reefs between conspicuous isopods and epibiotic microbial communities dominated by cyanobacteria, which give the isopods a bright red color and produce a chemical defense repulsive to fishes. For isopods, juveniles emerge directly from female brood pouches; because the mother hosts defensive microorganisms, ample opportunity exists for direct transmission of these ectosymbionts between isopods generations. In the field, even the smallest juveniles are covered by cyanobacteria. Among captive isopods, newly emerged juveniles, which hatch "naked," quickly become covered by the protective symbionts, suggesting that they behaviorally acquire them from their mother or neighboring isopods.

A most intriguing example of the involvement of a symbiont in the chemical protection of early life stages against predation is that of the bryozoan *Bugula*

neritina. Lopanik et al. (unpublished data) demonstrated that bryostatins protect larvae of *B. neritina* against predation by fish. Adults and larvae of this bryozoan host a unique bacterial symbiont, Candidatus *Endobugula sertula*, which is the putative source of the pharmacologically potent bryostatin macrolides (Davidson et al., 2001). Because the larvae carry only a small number of *E. sertula* cells (~3000 per larva) (Haygood et al., 1999), those carried by the larvae are not likely the source of the larval bryostatins. A much higher abundance of the symbiont occurs in the bryozoan's funicular cords, which transport nutrition between zooids and to the developing embryos, and in the ovicells where the larvae develop (Haygood, personal communication). Thus, a more plausible hypothesis for the origin of the larval bryostatins is that *E. sertula* in the funicular cords and the ovicells produce bryostatins that are transported by an unknown mechanism to the developing larvae.

SUMMARY

Given that bioactive natural products can deter feeding by diverse types of predators, the potential for these chemicals to diminish predation on early life stages seems great and, therefore, of major importance for understanding the evolution of life histories and reproductive characteristics of marine invertebrates. Life-history models incorporating parameters based on our developing understanding of early life-stage mortality, morphology, and behavior for individual species and their relationships to deterrent chemistry will yield more accurate hypotheses and predictions for investigating the population dynamics of many benthic marine invertebrates. For species with low larval mortality, population bottlenecks must occur at other life stages, most likely among populations of newly established juveniles, which, according to the hypothesis of Orians and Janzen (1974), may be constrained in their ability to elaborate a chemical defense.

While life-history models examine potential fitness advantages for incremental changes in various egg or larval characteristics (McEdward, 1997), chemical defenses have perhaps their primary impact on whether or not a larva can survive exposure to the intense predation common in many benthic habitats. For larvae of most species that can not produce a chemical defense, an exposed existence is unlikely in benthic habitats. Secondarily, if predation is not an issue for species with chemically defended larvae, then their larvae may be free to evolve characteristics, such as a short swimming duration and large size that minimize larval and juvenile mortality, respectively, by limiting dispersal away from suitable habitat and yielding larger juveniles immediately after settlement and metamorphosis.

As our knowledge of chemical defenses in early life stages of marine invertebrates grows, we will be able to examine connections among phylogeny, ecology, chemical defenses, and reproductive characteristics more rigorously. The results

of these analyses should provide a reasonable ability to confidently examine more general and far-reaching hypotheses about the roles of secondary metabolites in larval and juvenile ecology and for life-history evolution of marine invertebrates.

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MINI REVIEW

COMMUNITY AND ECOSYSTEM LEVEL CONSEQUENCES OF CHEMICAL CUES IN THE PLANKTON

MARK E. HAY[1,*] and JULIA KUBANEK[1,2]

[1]*School of Biology*
[2]*School of Chemistry and Biochemistry*
Georgia Institute of Technology
Atlanta, Georgia 30332-0230, USA

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Abstract—Aquatic organisms produce compounds that deter consumers, alter prey behavior, suppress or kill target and nontarget species, and dramatically affect food-web structure, community composition, and the rates and pathways of biogeochemical cycles. Toxins from marine and freshwater phytoplankton create health hazards for both aquatic and terrestrial species and can significantly affect human activities and the economic vitality of local communities. A reasonable case can be made that phytoplankton metabolites such as dimethyl sulfide (DMS) link interaction webs that span hundreds to thousands of kilometers and connect production from oceanic phytoplankton to desert cacti and coyotes via zooplankton, fishes, and sea birds. The possible role of DMS in global heat budgets expands this effect even further. The ecosystem-wide and potentially global consequences of aquatic chemical cues is an underappreciated topic that warrants additional attention.

Key Words—Aquatic chemical ecology, biotoxin, complex interactions, DMS, harmful algal bloom, phytoplankton, zooplankton.

INTRODUCTION

Chemical signals produced by aquatic organisms play important roles as deterrents of consumers, pathogens, and competitors and as cues used for foraging, reproduction, and assessing danger from a variety of natural enemies. Such direct

* To whom correspondence should be addressed. E-mail: mark.hay@biology.gatech.edu

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effects are becoming well documented in a variety of aquatic systems (Hay, 1996; Tollrian and Harvell, 1999; McClintock and Baker, 2001). In this review, we emphasize that these same chemical cues commonly have indirect and cascading effects on the ecology and evolution of entire communities and ecosystems. These larger-scale spatial and temporal effects are less well studied and understood. As such, they offer considerable intellectual challenges to, and opportunities for, enhancing our appreciation for how chemical cues produce cascading impacts on species, communities, and ecosystems beyond the initial group of directly interacting organisms. Given our charge to focus on novel and less well-known topics for this mini-review, we chose to emphasize chemical cues among planktonic organisms. Direct effects of chemical cues in these systems is poorly known relative to those documented in benthic systems (Hay, 1996; McClintock and Baker, 2001). Additionally, studies of chemically mediated planktonic interactions have rarely progressed to the point of unambiguously identifying the metabolites responsible. However, it is clear that chemically mediated interactions are omnipresent among the plankton and that chemical cues commonly produce cascading effects far beyond the initial participants. We discuss examples of this below.

PHYTOPLANKTON TOXINS IN LAKES

Given the smaller size and contained nature of lakes and ponds relative to oceans, ecosystem level effects of chemically mediated interactions could be most apparent in these systems. Indeed, there are numerous cases in which toxins produced by pelagic freshwater cyanobacteria have been shown, or suspected, to produce strong effects on both aquatic organisms and nearby terrestrial species, including humans. Cyanobacterial poisoning of animals has been reported since the 1800s. Kills have included fishes, birds, dogs, livestock, and 50 renal dialysis patients in Brazil (Jochimsen et al., 1998; Chorus, 2001). In many cases, liver-targeting cyanobacterial microcystins are believed to have caused mortality. Microcystins are contained within cyanobacteria cells and are not commonly released into the water. However, cell lysis releases microcystins, and these can persist in freshwater systems for several weeks because their cyclic structure and unusual amino acid composition renders them resistant to degradation by many common bacterial proteases. Thus, animals can be affected via consumption of cells or via water-borne exposure to these released toxins. However, despite the copious literature on microcystins and closely related compounds, numerous other assays and chemical investigations suggest that cyanobacteria produce many as yet unidentified peptides, depsipeptides, lactones, alkaloids, and other metabolites with broad-reaching effects, some of which may be responsible for the ecological consequences attributed to microcystins (Chorus, 2001).

Humans who have ingested water exposed to cyanobacterial blooms have experienced abdominal pain, nausea, vomiting, diarrhea, headache, fever, muscular

pain, sore throat, dry cough, blistering of the mouth, atypical pneumonia, and elevated liver enzymes (Chorus, 2001). Illnesses in Europe, Australia, and the United States have been tracked to toxic cyanobacteria, but the role of the particular compounds causing these symptoms has not been clearly demonstrated. Given these symptoms, humans alter their behavior once cyanobacterial blooms are detected. This includes prohibition of human uses [drinking, swimming, water-contact activities—especially for those with compromised livers (e.g., alcoholics, those infected with hepatitis B)] and treatment of drinking water by filtration, chlorination, sieving, floculation with chemicals, adjustment of pH, and ozonation.

Numerous investigations have also demonstrated the effects of cyanobacterial metabolites on aquatic organisms. Survival, growth, and time of hatching were modestly affected when fish embryos were exposed to ecologically realistic concentrations of dissolved microcystins; older fish exposed to microcystins exhibited altered levels of mobility and feeding and shifted activity between day- and nighttime patterns (Chorus, 2001). Given the dramatic trophic cascades that fish feeding produces in lakes (Carpenter et al., 1985), these altered behaviors may affect ecological patterns and processes throughout the entire body of water.

Some zooplankters (e.g., copepods) are discriminating feeders that select among phytoplankton particles based on characteristics including taste (Porter, 1977). In contrast, other zooplankters (e.g., *Daphnia*) feed more indiscriminately and do not appear to select based on flavor or other chemical signals (DeMott, 1986; DeMott et al., 1991). Because they are more willing to feed on the foods that are offered, these indiscriminate feeders offer unusual opportunities for assessing the consequences of consuming toxic prey. Lampert (1982) surveyed how the presence of the toxin-producing cyanobacterium *Microcystis aeruginosa* affected 13 species of cladocerans and demonstrated that the smaller species were less affected than the larger ones. He speculated that this differential tolerance could drive seasonal shifts in species composition within the zooplankton community. Differential effects of the toxic cyanobacteria *Anabaena affinis* on cladocerans and rotifers can also reverse competitive outcomes among zooplankters and may change the species composition and size structure of zooplankton communities (Gilbert, 1990; Kirk and Gilbert, 1992). An overview of recent investigations (Christoffersen, 1996) suggests that cyanobacteria: (1) affect both phytoplankton and aquatic macrophytes via allelopathic compounds, (2) disrupt linkages between phytoplankton production and zooplankton growth, and (3) shift rates and pathways of energy flow, nutrient cycling, and degradation from pelagic to benthic zones. Thus, phytoplankton metabolites appear to produce cascades throughout the food web, affecting not only community structure and species composition, but also fundamental pathways and rates of biogeochemical cycles.

As might be expected from the strong effects noted above, cyanobacteria that produce toxic compounds can exert strong selection on herbivores, altering their genotypes over relatively short periods of time. As an example, Hairston et al.

(2001) hatched eggs of *Daphnia galeata* that had been deposited in stratified lake sediments over a span of 35 years; the prevalence of planktonic cyanobacteria had increased substantially during this 35-year period. *Daphnia* representing genotypes from three distinct deposition times were fed either a palatable alga, or a mix of the palatable alga plus the toxic cyanobacterium *Microcystis aeruginosa*. Effects of the cyanobacterium on the performance of each genotype demonstrated that *Daphnia*'s ability to tolerate the cyanobacteria had increased within little more than a decade.

Phytoplankton may also respond to, or be chemically manipulated by, other phytoplankton. Keating (1977) monitored bloom sequence in a lake over a three-year period, then tested the effects of cell-free filtrates of dominant cyanobacteria on other phytoplankters that either preceded or followed the cyanobacterial bloom. Filtrates suppressed predecessors in five of eight trials (no effect in the other three) while enhancing successors in six of nine trials (no effect in the other three). This suggests that allelopathic agents may affect the sequence of blooms in lakes, but the involvement of particular compounds has not been demonstrated.

PHYTOPLANKTON TOXINS AND EFFECTS IN THE SEA

Phytoplankton dominate primary production in the world's oceans and are found in almost all marine habitats. Approximately 7% of phytoplankton species are known to form large-scale blooms, dramatically impacting marine communities. Of these, about one third have been documented to produce noxious compounds that kill marine organisms or that are subject to trophic transfer and bioconcentration in animals (Smayda, 1997). The tendency of phytoplankton to form blooms, coupled with their ability to be moved over hundreds of kilometers and to produce potent toxins, can result in massive wildlife kills and shutdowns of coastal tourism and fisheries, dramatically impacting local economies and the health and function of coastal ecosystems. The increased frequency and wider geographic distribution of toxic algal blooms (Anderson, 1989) have become major environmental and health concerns around the world (NRC, 1999). Many phytoplankton toxins are passed to higher trophic levels, eventually reaching fishes, marine mammals, and human consumers (Shumway, 1990). Animal kills worldwide have been attributed to phytoplankton compounds with diverse molecular structures including polycyclic polyketides, macrolides, isoprenoids, and amino acid-derived compounds produced by dinoflagellates, diatoms, and other microalgal taxa (Figure 1). Much remains to be learned about how blooms initiate, why populations of phytoplankton expand so rapidly, how they are affected by interactions with other organisms, and why blooms stop. The crucial ecological question of why many microalgal species produce potent neurotoxins remains unanswered, although these compounds clearly have dramatic consequences

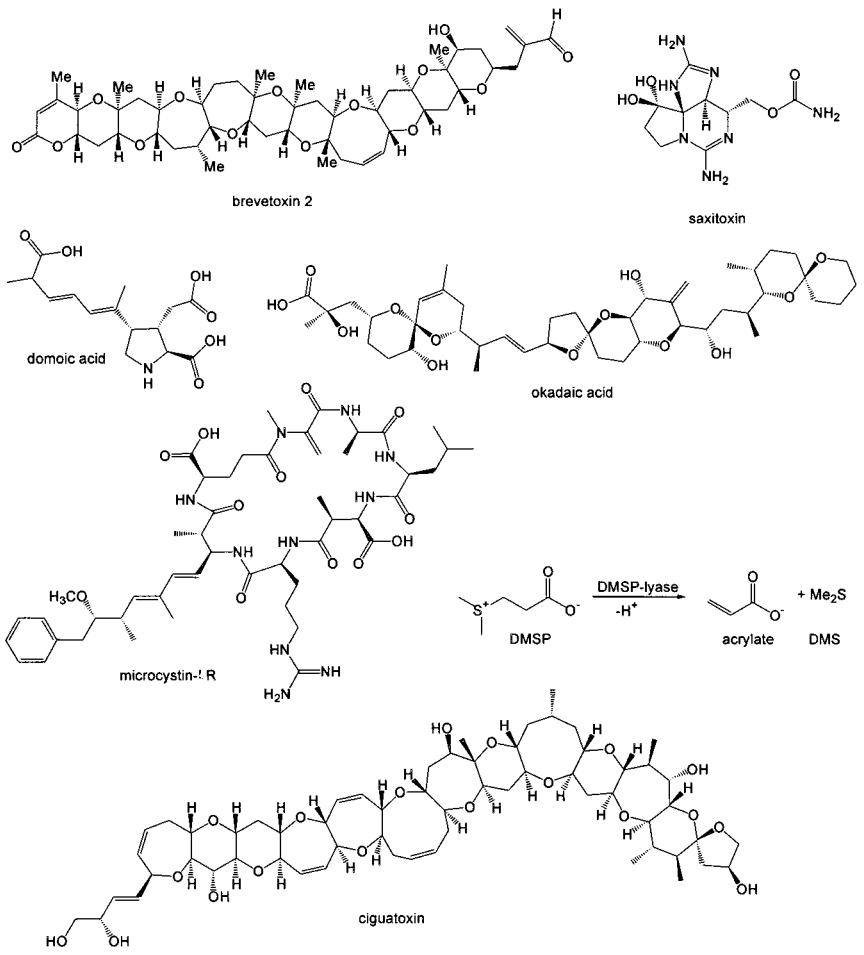


FIG. 1. Microalgal metabolites that affect food-web structure, community composition, human health and economy, and/or the rates and pathways of biogeochemical cycles.

on populations of many marine species, on community structure, and often on ecosystem function.

Microalgal blooms that involve transfer of algal metabolites to fish or filter-feeding shellfish, that cause animal mortality, and/or that reduce coastal water quality are referred to as harmful algal blooms (HABs) because of their negative economic and human health consequences. In the United States, conservative estimates of direct and indirect costs due to HABs averaged $49 million per year for the period 1987–1992, including public health, commercial fishery,

recreation/tourism, and monitoring/management costs [reported in year 2000 US dollars (Anderson et al., 2000)]. Ciguatera poisoning, the most prevalent human illness caused by ingestion of HAB toxins, sickens 50,000 people in tropical countries each year, and the reported numbers are believed to represent only 20–40% of the actual cases (NRC, 1999). Ciguatera toxins such as ciguatoxins, maitotoxin, and gambieric acids are produced by benthic dinoflagellates such as *Gambierdiscus toxicus* that grow epiphytically on seaweeds and on coral rubble. The toxins are bioconcentrated through the food web as herbivorous fishes and invertebrates feed on seaweeds and small turf communities harboring the dinoflagellates. Toxins consumed by the herbivores are then transferred, via predation, to larger fishes such as moray eels and barracuda. During the process of bioconcentration, the parent metabolites may be altered to become more toxic, generating greater risk for humans consuming top carnivores from reefs (Yasumoto and Murata, 1993). Many species of tropical fishes accumulate these toxins without showing signs of physiological stress; however, when these fish are consumed by humans, ciguatera toxins activate voltage-gated sodium channels, causing gastrointestinal and respiratory distress, followed by neurological problems, bone and joint pain, and chronic fatigue that can last for months or years. Because the distribution of ciguateric dinoflagellates is geographically patchy but generally persists over time, fishermen and local residents often know which reefs harbor contaminated fishes.

There has been a strong association between large-scale animal kills and blooms of several pelagic microalgal species, such as *Karenia brevis* (ex *Gymnodinium breve*), which produces brevetoxins. Immunohistochemical and pathological data have supported the role of brevetoxins in the death of animals such as manatees (Bossart et al., 1998), and strong circumstantial evidence points to brevetoxins as the cause of death for dolphins, pelicans, and various fishes and invertebrates during several blooms over the last century (Gunter et al., 1948; Steidinger et al., 1973).

Toxins produced by HABs can fundamentally affect entire marine ecosystems, can impact locations hundreds or thousands of kilometers from where blooms initially form, and can even affect human activities and terrestrial ecosystems. As an example, in 1987–1988, satellite images showed a bloom of *Karenia brevis* initiating along the Gulf coast of southern Florida, being transported northward by the Gulf Stream, and being delivered to inshore North Carolina by a loop current from the Gulf Stream. *K. brevis* had never before been seen so far north of the tropics. This unanticipated bloom had a variety of deleterious effects including: (1) massive scallop mortality in coastal North Carolina, (2) closure of shellfish harvesting, (3) respiratory distress in humans and pets along coastal beaches with 48 documented cases of human illness attributed to brevetoxicosis, and (4) reduced coastal tourism and recreation by humans; costs to coastal communities were estimated at $24 million due to loss of the shellfish harvest alone (Tester and Fowler, 1990).

The effects of HABs on coastal resources and on charismatic megafauna such as whales, dolphins, and manatees are relatively well documented compared to their potential effects on less obvious systems such as detrital food webs and soft-sediment communities (e.g., Gunter et al., 1948; Kvitek, 1993; Bossart et al., 1998). When toxic blooms senesce, do their natural products affect detrital pathways or biogeochemical cycles and rates of processing? The direct effects of compounds are unknown, but faunal changes do occur in soft-bottom communities beneath toxic algal blooms. When Olsgard (1993) monitored soft-bottom communities at depths of 69–184 m and described changes in fauna over a five-year period that contained a bloom of the toxic flagellate *Chrysochromulina polylepis*, he found a bloom-associated depression in numbers of both species and individuals; this effect persisted for nearly two years following the bloom. Although such contrasts confound the effects of phytoplankton toxins with the potential effects of increased detrital biomass and decreased light/oxygen, they suggest the potential for substantial chemically mediated effects on detritivore communities.

Diatoms belonging to the genus *Pseudonitzschia* also have cascading effects on coastal food webs. On both the east and west coasts of North America, these diatoms have been shown to produce domoic acid, a neuroexcitatory amino acid that enters the food web through shellfish such as mussels (Bates et al., 1989) and filter-feeding fishes such as anchovies (Scholin et al., 2000). Humans, sea lions, cormorants, and other consumers of toxic fish or shellfish have all been killed by domoic acid poisoning in episodes that have only been documented in the last 15 years. Domoic acid kills mice at concentrations similar to those found in contaminated seafoods, and the toxin was isolated from the body fluids of animals that died after consuming these foods, providing strong support for toxin-induced mortality (Bates et al., 1989; Scholin et al., 2000).

When neurotoxic dinoflagellates such as *Alexandrium* spp. bloom in coastal waters, they are consumed by filter-feeding animals such as butter clams. These clams are relatively unaffected by consuming the toxins because their neurons are insensitive to saxitoxin and other closely related sodium channel-blocking toxins produced by *Alexandrium* spp. (Kvitek, 1993). Saxitoxins are concentrated in clam siphons where small fish can detect them during "siphon-nipping" predatory behavior, leading the fish to avoid toxic clams. Siphon-nipping behavior by fishes may have selected for the sequestration of toxins in these more exposed regions of the clams. When clam toxicity levels are high, sea gulls and sea otters reduce their overall feeding on clams and reject clam siphons. Thus, the microalgal toxins are transferred from primary producers to clams, where they act as a chemical defense against the next trophic level, deterring both aquatic (otters and fishes) and terrestrial (birds) vertebrate predators. Because otters avoid toxic shellfish and suffer physiological distress if they mistakenly feed on them, the dinoflagellate blooms that occur primarily in protected bays may historically have restricted otters from these bloom areas and limited the feeding activities of these keystone

species to outer coasts where clams have lower concentrations of dinoflagellate-derived toxins (Kvitek, 1993). Because otter feeding has dramatic cascading effects on nearshore ecosystems, turning unproductive urchin barrens into productive and species-rich kelp forests (Estes and Duggins, 1995), restriction of otters due to microalgal toxins has the potential to fundamentally alter the distribution, abundance, and function of entire coastal ecosystems (Duggins et al., 1989, Kvitek, 1993). Consequently, these phytoplankton metabolites have cascading effects on community structure and on temporal and spatial ecosystem dynamics involving seaweeds, invertebrates, fishes, and keystone predators such as otters.

Additionally, recent work by Bricelj et al. (2000) has shown that some bivalves show differential susceptibility to the accumulation of dinoflagellate toxins and to their negative physiological effects. In experiments in which softshell clams (*Mya arenaria*) from two populations were exposed to toxic *Alexandrium tamarensis*, clams from the population with no previous history of exposure to *Alexandrium* showed higher mortality, lower respiration, impaired burrowing capacity, reduced food clearance rates, and a 10-fold lower toxin accumulation, relative to clams from a historically exposed location. Failure of clams to burrow could have negative consequences for toxin-susceptible individuals, because exposed bivalves are more vulnerable to predatory birds and crustaceans. Thus, exposure to occasional blooms of toxic *Alexandrium* may lead to increases in the number of toxin-resistant individuals in a population, due to differences in fitness between resistant and susceptible bivalves. Ongoing changes in the frequency and geographic distribution of toxic microalgae may result in further selection for tolerance of toxins among filter-feeding organisms whose populations show variance in their degree of resistance to toxins. This could produce a gradual increase in the levels of toxins contained in bivalves that humans harvest. The mechanism(s) by which some bivalves minimize the effects of phytoplankton toxins are as yet unknown, although the possibility of resistance-conferring mutations in mollusk sodium channels is the subject of current investigation (V. M. Bricelj, personal communication).

Although phytoplankton metabolites can enter food webs via shellfish and filter-feeding fishes, phytoplankton face the greatest threat of herbivory from grazing zooplankton including ciliates, copepods, rotifers, tintinnids, and invertebrate larvae (Turner and Tester, 1997). The greatest opportunity for chemically mediated interactions may therefore occur between phytoplankton and zooplankton. Nevertheless, toxicity of phytoplankton compounds has generally been determined against vertebrates rather than zooplankton, with effects on zooplankton being uncertain. Zooplankters sometimes prefer, or perform better on, nontoxic versus toxic species or clones (e.g., Huntley et al., 1986; Teegarden, 1999). However, correlations such as these can be confounded by other microalgal characteristics, including differences in morphology, nutritional value, and chemicals other than known neurotoxins. Purified phytoplankton compounds added to seawater containing copepods have occasionally been found to negatively affect zooplanktonic grazers (e.g.,

Miralto et al., 1999), although it is unlikely that this method adequately represents the consumption of compounds as part of a natural diet. Rigorously documenting how zooplankters are affected following consumption of particular compounds may demand further technological developments. Despite uncertainty regarding the effect of phytoplankton metabolites on zooplankton behavior and physiology, a handful of studies have shown that some zooplankton (e.g., copepods) do accumulate phytoplankton metabolites such as brevetoxins (Tester et al., 2000). Thus, zooplankton provide another route whereby phytoplankton toxins can enter the food web and reach higher consumers such as fishes, whales, and humans. If zooplankters avoid feeding on phytoplankters due to their production of specific compounds or selectively graze on nontoxic phytoplankton (favoring the proliferation of toxic phytoplankton), then phytoplankton compounds may play a crucial role in the initiation and persistence of large-scale harmful algal blooms.

Additionally, toxic microalgae may proliferate due to the allelopathic effects of their excreted metabolites on competing microalgae. For example, in cross-culturing experiments, medium preconditioned by the benthic dinoflagellate *Prorocentrum lima* reduced growth of diatoms (Windust et al., 1996) and other epiphytic dinoflagellates (Sugg and VanDolah, 1999). Windust et al. (1996, 1997) reported that okadaic acid, a product of *P. lima* that inhibits protein phosphatases, depressed diatom growth, and they proposed that less toxic sulfated and diol esters are rapidly biotransformed to okadaic acid upon release from *P. lima* cells and/or upon contact with competing cells. *P. lima* was found to be considerably less sensitive to okadaic acid than were other microalgae. In contrast, Sugg and VanDolah (1999) showed that although protein phosphatase activity of competing dinoflagellates was inhibited by *P. lima* exudates and by pure okadaic acid, reduced growth of competitors was associated with a nonokadaic acid fraction.

PREY RESPONSES TO CHEMICAL CUES FROM CONSUMERS

Many marine and freshwater zooplankton undergo diel vertical migration (DVM), usually living at darker depths during the day and feeding in surface waters at night when their visually foraging predators are less active. This behavior is cued by chemical detection of zooplankton predators (De Meester et al., 1999). DVM apparently lessens zooplankton susceptibility to consumers, but the (as yet unknown) compounds mediating this behavior also affect biogeochemical processes systemwide. As an example, in marine systems, about 15–50% of all metazoan zooplankton biomass above 500 m moves to near the surface at night and migrates to depths of 300–600 m during the day (Longhurst and Harrison, 1988). Given the permanent density discontinuity at 40–200 m in tropical and subtropical oceans, this migration can transport carbon and nutrients below the pycnocline, mediate the alkalinity of surface waters, and influence CO_2 uptake at the surface. As zooplankters migrate from the photic zone to below the pycnocline, assimilated

carbon and nutrients move with them. Some of this stays in the deeper waters due to respiration, excretion, and predation by deep-water consumers. It is estimated that the flux of respired carbon due to DVM equals 13–58% of computed particulate sinking flux across the pycnocline and adds 5–20% to current estimates of global sinking flux of organic carbon across the pycnocline (Longhurst et al., 1990).

Available nitrogen often limits production in the photic zone, and DVM further lowers available nitrogen. Crude calculations indicate that exudation of nitrogen from zooplankton performing DVM may move 21% of the total nitrogen pool below the photic zone (Longhurst and Harrison, 1988). Thus, when zooplankton detect chemical evidence of nearby consumers and initiate diel vertical migrations, their behavior moves significant quantities of both carbon and nitrogen from the photic zone to below the pycnocline, affecting both biogeochemical fluxes and the structure of marine food webs. Given the smaller and more contained nature of lakes versus oceans, these chemically cued processes might produce even stronger effects in stratified freshwater systems. However, because lakes are more prone to climatic disturbance, the stratification of nutrients could be "reset" more frequently.

Just as zooplankton chemically sense their consumers and undertake DVM, some phytoplankton chemically sense zooplankton grazers and respond in adaptive ways. Solitary cells of phytoplankton such as *Scenedesmus acutus* often form colonies that are more resistant to grazing if they are exposed to water conditioned by feeding *Daphnia* (Van Donk et al., 1999). Rather than inducing increased resistance, dinoflagellates use chemical indicators of zooplankton presence to determine when to excyst and emerge from the sediment "seed bank" into the water column. When Hansson (1996) incubated sediment cores in containers with no zooplankton, zooplankton in a cage so that they could not access the emerging phytoplankton, or dead zooplankton (presenting the chemical signals from *Daphnia*, but not their feeding action), a sixfold greater emergence was found in the absence of zooplankton than in either other treatment. In field assays, both *Peridinium wisconsinense* and *P. pusillum* emerged in greater numbers from sediments in lakes with small zooplankon populations than ones with larger populations. Additionally, like zooplankton, some species of mobile phytoplankton also undergo diel vertical migration, often moving to deep waters when their consumers move to shallow waters (Jones, 1993). Thus, (as yet unknown) chemical cues from zooplankters appear to be perceived by microalgae, producing effects on seasonality, succession, and composition of phytoplankton communities (Rengefors et al., 1998).

CHEMICALLY CUED CASCADES OVER THOUSANDS OF KILOMETERS?

Microalgal metabolites that function as defenses against ciliate or zooplankton consumers have the potential to create cascades of interactions that span

hundreds or thousands of kilometers and involve species and interactions that are far removed from the initial participants. One of the most interesting examples of this is the cascading effect that phytoplankton-generated dimethylsulfide (DMS) and acrylate appear to have on ciliates, zooplankton, fishes, sea birds and, potentially, desert plants, desert animals, and global weather patterns.

Many grazers selectively avoid, or less heavily utilize, phytoplankton strains that have high potential to cleave dimethylsulfoniopropionate (DMSP) enzymatically to DMS and acrylate when ingested by grazers (Figure 1) (see review by Wolfe, 2000). This potential appears to be regulated more by levels of the enzyme DMSP lyase than by levels of DMSP. The biochemical nature of the variance in DMSP lyase is not known, but consumers like ciliates and dinoflagellates avoid strains with high ability to create DMS and acrylate from DMSP, relative to strains with lesser ability to do this. DMS should rapidly diffuse from consumers, potentially providing a cue for other organisms. Acrylate sterilizes consumer guts and cascades through food chains from common phytoplankton such as *Phaeocystis*, to dominant zooplankton like euphausids, and finally to higher level consumers such as penguins, with antimicrobial effects in the guts of both the primary and secondary consumers (Sieburth, 1960).

In areas of high productivity, significant quantities of DMS are released into surface waters, held in benthic pore waters beneath areas of high productivity, and released into the atmosphere (Andreae, 1985). There are numerous examples of atmospheric DMS being elevated three- to sixfold over background levels near productive areas such as polar frontal zones. Thus, DMS is often associated with areas of high productivity, areas where planktivores are feeding, areas of increased krill density, and areas where fishing success by birds is likely to be greater.

There is mounting evidence that certain species of sea birds with strong olfactory capabilities use DMS as a foraging cue and that the use of an area by these birds sets up a cascade of increased use by other birds that do not have olfactory systems that are as highly developed. Nevitt (2000) provides a convincing overview of this topic. Certain tube-nosed seabirds (procellariiformes) have among the largest olfactory bulbs of any birds and forage over hundreds and even thousands of kilometers of featureless ocean searching for patchily distributed foods such as krill, squid, and fish. Field tests have shown that these birds are attracted to fishy odors (cod liver oil, tuna oil, fish homogenate) and that a specific subset of the smaller and more cryptic species that have especially strong olfactory capabilities are attracted to DMS dispersed in either water or air (Nevitt et al., 1995). For many organisms that migrate over long distances and use odors as cues, the notion of homing to odors produced hundreds or thousands of kilometers away seems unlikely due to turbulence disrupting odor plume gradients and due to dilution dropping signals below detection limits. Nevitt notes that DMS signals from damaged phytoplankton can persist for hours to days and suggests that birds use DMS associated with upwelling areas, seamounts, or other productivity

discontinuities, as olfactory guideposts to aid navigation to general fishing areas. Once in productive areas, they may use more localized searching strategies.

A second strategy for these birds is to fly directly from the colony to feeding grounds that are hundreds or thousands of kilometers away. To do this, procellariiformes may rely on spatial memory, experience, olfactory guideposts, or other navigational cues associated with persistent olfactory signals along bathymetric features. Numerous species of albatross and the white-chinned petrels seem to use this strategy, flying directly to distant feeding grounds without foraging along the way. These feeding grounds are not constant, but can fluctuate in space and time; therefore, birds may start area-restricted foraging only after changes in olfactory cues indicate a productive fishing area (Nevitt, 2000).

On research cruises where DMS and bird densities were recorded, blue petrels and prions were significantly associated with areas where DMS levels were highest (Nevitt, 2000). DMS is a predictor of krill grazing, and petrel and prion diets are composed predominantly of crustaceans, including krill, amphipods, and copepods. Surveys of areas with high, medium, and low DMS levels indicated that prions were the primary users of high DMS level sites. At medium- and low-concentration sites, prions were present, but there were also approximately 15 additional species of birds. This pattern suggests that high DMS sites may be new patches that erode over time. It seems possible that prions use superior olfaction to find sites first and specialize on these until competing birds, or predatory birds, become common. Later-arriving species seem to be less sensitive to DMS signals and may visually cue on foraging prions to find more productive sites. This is consistent with findings that smaller, more cryptic species such as prions and storm-petrels respond strongly to DMS-scented slicks or aerosols, whereas larger, more visible species do not (Nevitt et al., 1995). This process may link local production of phytoplankton chemical cues to abundant zooplankton and fishes, and then to birds and terrestrial systems that are hundreds of kilometers distant. As seabirds move marine biomass and nutrients to nesting sites on distant terrestrial habitats, they are providing nutrient and energy subsidies to these terrestrial systems. Such marine inputs can fundamentally alter resource-starved terrestrial systems by producing demonstrable, and often dramatic, increases in the growth rates or densities of organisms as diverse as desert cacti, coyotes, beetles, and spiders (Polis and Hurd, 1996; Rose and Polis, 1998; Sanchez-Pinero and Polis, 2000). The DMS affecting this transfer of energy and nutrients can even be argued to produce broader-scale effects, in that atmospheric oxidation products of DMS are important in the formation of aerosols in the lower atmosphere, affecting cloud formation, altering the earth's albedo, and impacting global heat balance (Charlson et al., 1987).

The cross-ecosystem effects of aquatic chemical cues that we describe here should not be unique to the plankton. Such interactions probably occur in numerous systems, but are simply underinvestigated. Leaves falling from trees into

forest streams are one of the primary energy sources for these streams, but little is known about how specific metabolites contained in these terrestrial leaves affect aquatic systems in terms of rates of degradation, use by shredding insects, or stream food webs and biogeochemical cycles in general. Similarly, when large masses of chemically defended seaweeds are torn from tropical reefs and deposited on deep sand or mud plains, do the metabolites in these tissues affect microbial processes, biogeochemical cycles, or the species normally using these sedimentary environments? Chemical signals directed at target organisms in one community may commonly affect nontarget species in other systems—this possibility seems worthy of further attention by experimentalists.

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INFLUENCE OF AGE AND BODY SIZE ON ALARM RESPONSES IN A FRESHWATER SNAIL *Pomacea canaliculata*

KATSUYA ICHINOSE[1,*]

National Agricultural Experiment Research Center for Kyushu Okinawa Region
Suya 2421, Nishigoshi
Kikuchi-gun, Kumamoto-ken, 861-1192 Japan

[1]*Present Address: IRBI, CNRS UMR 6035*
Institut de Recherche de Biologie de l'Insecte
Faculté des Sciences et Techniques
Parc de Grandmont, 37200 Tours, France

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Abstract—A hypothesis that size selection of prey by predators elicits size-specific responses from prey was examined. Freshwater snails, *Pomacea canaliculata*, ages 1, 3, 7, 15, 30, or 60 days, were given an extract of 3-day-old snails, and 3-day-old snails were given extracts of snails of the other ages or eggs. Snails 15 days or younger crawled out of the water in response to the 3-day-old snail extract, but older ones did not. The 3-day-old snails responded to the extracts of snails of all examined ages, but not to the extract of eggs. Snails of four size classes, 3-days-old, small (shell lengths 8–12 mm), medium (13–20 mm), and large (>28 mm) were given extracts of snails of each of these four classes. The 3-day-old snails crawled out of the water in response to the extract of 3-day-old snails, but showed a lower or no response to other extracts. Larger snails buried themselves in the soil in response to the extract of snails of similar sizes. These responses are discussed in the context of the evolution of the snail's avoidance behavior in response to the size-dependent prey choice by the predator.

Key Words—Alarm response, apple snail, crawling, ontogenetic differentiation, *Pomacea canaliculata*, predator-prey relationship, self-burying.

INTRODUCTION

Prey use chemical cues to detect predators (Dodson et al., 1994; Chivers and Smith, 1998; Kats and Dill, 1998; Zimmer and Butman, 2000). Some anurans also use mechanical stimuli (Warkentin, 1995; Chivers et al., 2001). Peptides, disaccharides,

* E-mail: ichis@affrc.go.jp

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and ammonium ions have been identified as cues (Rittschof, 1990; Kats and Dill, 1998; Zimmer et al., 1999; Rahman et al., 2000). These cues are effective only at low concentrations (Petterson et al., 2000; Von Elert and Pohnert, 2000). Selection pressure by predators causes prey to change their life history strategy (Skelly and Werner, 1990; Buskirk, 2000; Lardner, 2000). Prey can respond to predators in several ways (Boersma et al., 1998), although local populations may have fewer responses than the species (Laurila, 2000; Von Elert and Pohnert, 2000). The prey's response depends on how the predator attacks.

The presence of predators such as crayfish and fish causes freshwater snails to seek habitats where predator pressure is weaker (Turner et al., 1999, 2000; Lewis, 2001). Snails can escape predators by crawling out of the water (Alexander and Covich, 1991a,b; Covich et al., 1994; Lewis, 2001) or by self-burying in the soil (Snyder and Snyder, 1971). Crawling out of the water avoids small predators, such as dragonfly larvae, small fish, or crayfish (Rowe, 1987; Slootweg, 1987; Cook, 1989), but exposes snails to bigger predators, such as birds (Beissinger, 1983; Donnay and Beissinger, 1993). Therefore, snails that are subject to predation by bigger predators need to respond differently. Hence, snails may have evolved size-dependent avoidance behavior (Covich et al., 1994; Dodson et al., 1994).

Growth- or size-dependent avoidance behaviors by snails have rarely been reported (e.g., Watt and Young, 1992; Mondor et al., 2000). Snyder and Snyder (1971) reported that *Pomacea paludosa* adults bury themselves to avoid predators. Halwart (1994) noticed that small *P. canaliculata* snails crawled above the water surface in an aquarium when they were maintained with common carp, an effective predator. Thus, snails may change their avoidance behavior as they grow. This hypothesis was tested by using *P. canaliculata* (Lamarck) (Gastropoda: Ampullariidae) of various ages and sizes, and the difference in avoidance behaviors is discussed in relation to the behavior of predators.

METHODS AND MATERIALS

Effects of Aging on Juvenile Snails. Six egg masses of *P. canaliculata* were collected in September to October 1999 in Saga, southern Japan. Each egg mass was kept individually in a Petri dish (90 $\times$ 20 mm) in the laboratory at 24 $\pm$ 1°C under a lighting regime of 16 hr light–8 hr dark (16L:8D). More than 1000 hatchlings emerged from each egg mass on 13 October. The snails from each egg mass were cultured in an aquarium (225 $\times$ 160 $\times$ 210 mm) in 2 liters of water. The water was changed every day, and then the snails were fed a spoonful of ground artificial bait for carp (Nippon Pet Food Co. Ltd., Tokyo, Japan). Dead hatchlings were removed when the water was changed. At 1, 3, 7, 15, 30, and 60 days after hatching, 60 snails were taken randomly from each aquarium. These snails are referred to as

age-varied snails hereafter. Also on each of these days, 360 3-day-old snails of an additional egg mass were used. These 3-day-old snails are referred to as age-fixed snails hereafter.

On each experiment day, 10 age-varied snails from each egg mass were transferred to each of six test tubes with 50 ml deionized water. Similarly, 10 of the age-fixed group were placed in each of 36 test tubes. Thus, 72 test tubes (36 for the age-varied group and 36 for the age-fixed ones) were prepared on each experiment day. The test tubes were kept in the laboratory at $24 \pm 1^{\circ}$C. One hour after transfer, 3–10 more age-varied and -fixed snails were taken from each aquarium and air-dried for 20 min. The weight of these snails, pooled by each egg mass, was measured to the nearest 0.001 mg. Snails from the aquarium were macerated in 0.001 M EDTA in a volume 1000 times their weight in a Petri dish. The supernatant is referred to as the extract. The maceration mimicked chewing of the snails by predators. Immediately after the extracts were prepared, the number of snails above the water surface (a_1) in each test tube was counted, and 0.5 ml of extract was gently dropped onto the water surface. All test tubes of the age-varied snails (receiver) received the extract of the age-fixed snails (sender), and each of the six tubes of the age-fixed snails received the extract of one of the age-varied snails. Thus, both receiver and sender were replicated six times for each egg mass on each experiment day. Application of 0.5 ml of EDTA alone evoked no significant response by snails. At 1 hr after the extract was added, the number of hatchlings above the water surface (a_2) was counted. The response (Rp) in each test tube was evaluated as:

$$Rp = (a_2 - a_1)/(10 - a_1). \quad (1)$$

Because only crawling out of the water was tested in this experiment, Rp was defined as 0 when $a_2 \leq a_1$ in a given test tube. Because a_1 was always ≤ 10, Rp was always 0. After the observation, the snails in each test tube were air-dried in the laboratory for 20 min and weighed to the nearest 0.01 mg. Means of arcsine-transformed Rp were compared by a two-way ANOVA for egg mass (random factor) and snail age (fixed factor).

Multiple-regression analyses of the hatchlings' response against mean weight of receiver or sender and age were performed. It was assumed that the hatchling's response to the extracts would be reduced as both the receivers and senders grew and their mean weight increased.

Response of 3-Day-Old Snails to Egg and Adult Snails. In a second experiment, 360 3-day-old snails of one egg mass from Saga were prepared as in the first experiment. Newly laid egg masses (30×10 mm) and adult females and males also were collected on this day from a canal in Kumamoto-ken, southern Japan. The experiment was performed in April to June 2000. As in the first experiment, 36 test tubes were prepared, and, 1.5 hr later, six tubes were each given 0.5 ml of either 0.001 M EDTA or extracts of 3-day-old snails, egg mass, adult females,

or males, or nothing. One hour later, the snail response was evaluated as before. The experiment was performed seven times. Mean responses were compared by a two-way ANOVA among the egg masses as a random factor and among the extracts as a fixed factor.

Comparison of Responses between Juvenile and Fully Grown Snails. In a third experiment, egg masses and snails of various sizes were collected from August to September 2000 in rice fields in Kumamoto-ken. The egg masses were placed in Petri dishes (90 × 20 mm) and kept in an incubator at 24 ± 1°C and 16L:8D. Snails of four size classes were used: 3-day-old snails, small snails (shell lengths 8–12 mm), medium (15–20 mm), and large (28 mm). Up to 100 snails of each class were placed in an aquarium (190 × 125 × 120 mm), that was submerged in a deeper aquarium (4 × 2 × 0.5 m) with 30 cm soil on the base, above which water was kept 10 cm deep. Females and males of the large class were put in different aquaria of the same size. Seven days before assumed hatching of the collected egg masses, 12 snails of each size class were taken from the snail stock and separately put in an aquarium (225 × 160 × 210 mm) with 2 liters of water. Snails hatched from egg masses were maintained as in the previous experiment. The experiment was replicated 18 times.

On the morning of the third day after hatching, 10 aquaria (225 × 160 × 210 mm) with 2 cm of soil and 2 liters of water were prepared. About 2 hr later, three 3-day-old snails and one snail of each size class were placed in each aquarium. One and a half hours later, 20 more 3-day-old snails and one to two snails of each of small to large classes were taken from the stock and air-dried for half an hour. The snails were weighed, and extracts of each class were prepared as before. The numbers of snails on the soil or wall in the water, on the wall above the water, buried in the soil, and floating beneath the water surface were counted. Then, 20 ml of 0.001 M EDTA or extract was given to each aquarium. Pairs of aquaria received the same extract, and the results of each pair were pooled. Snail behaviors were observed at 0.25, 0.5, 1, 2, 12, and 24 hr after addition of the extracts. Crawling above the water, floating beneath the water surface, and burial in soil were considered avoidance behavior. Resting on the soil or walls or closing the operculum were considered nonresponding behaviors. The avoidance response to a given extract at a given time was evaluated as:

$$Rp = (b_2 - b_1)/(c - b_1), \tag{2}$$

where b_1 and b_2 are the numbers of snails showing avoidance behavior before the extract was applied and at a given time, respectively, and c is the total number of snails in the paired aquaria, i.e., 6 for the 3-day-old snails, 2 for the small, medium, female and male snails, and 4 for large snail (female + male). The means of *Rp* in each snail class were compared among extracts by one-way ANOVA.

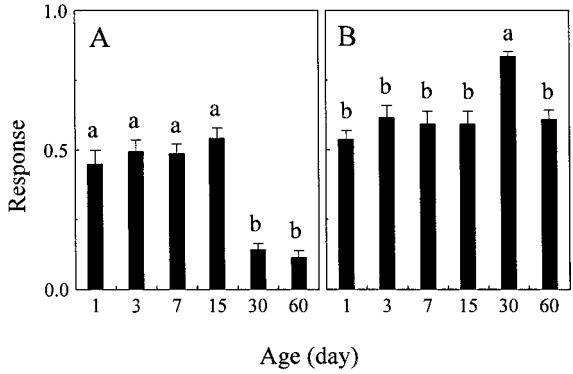


FIG. 1. Responses of 1-to 60-day-old snails to extract of 3-day-old snails (A) and those of 3-day-old snails to extracts of 1-to 60-day-old snails (B). Columns and bars denote the mean and 1 SE, respectively. Letters above the bar in each panel indicate significant difference at $P < 0.05$ by a Tukey's test.

RESULTS

Effects of Aging on Responses of Juvenile Snails. The age-varied snails reduced their responses to the extract of 3-day-old snails after the snails reached 30 days old (Figure 1A). The responses were different among egg masses ($F_{5,25} = 9.201$, $P < 0.001$) and among ages ($F_{5,25} = 23.150$, $P < 0.001$), but the interaction of these factors was not significant ($F_{25,180} = 1.547$, $P = 0.055$). A Tukey's test revealed differences at $P < 0.05$ in responses between 1–15 days and after 15 days, but the response was not different either within 1 to 15 days or 30 and 60 days.

Responses of the age-fixed snails were less varied (Figure 1B). The response was different among ages ($F_{5,25} = 12.512$, $P < 0.001$), but not among egg masses ($F_{5,25} = 2.211$, $P = 0.085$), and the interaction of these factors was significant ($F_{25,180} = 1.901$, $P = 0.009$). A Tukey's test revealed a difference at $P < 0.05$ between the response to the extract of the 30-day-old snail and the responses of the extracts of snails of the other ages, among which differences were not detected.

Multiple-regression equations for the response of the age-varied snails to 3-day-old snail extract and that of the age-fixed snails to extracts of age-varied snails were, respectively,

$$Rp = -0.0085a + 0.0014w + 0.5099, \quad r^2 = 0.250 \tag{3}$$

$$Rp = 0.0014a + 0.0001w + 0.6002, \quad r^2 = 0.015 \tag{4}$$

Where *Rp* is the response, *a* is the snail age on the experiment day, and *w* is the snail weight. The coefficient of age in equation 3 was significant ($F_{1,213} = 12.072$, $P < 0.001$), but that of weight was not ($F_{1,213} = 0.284$, $P = 0.595$). The coefficient of either variable in equation 4 was not significant ($F_{1,213} = 0.631$, $P = 0.428$ in age or $F_{1,213} = 0.000$, $P = 0.984$ in weight). The results indicate that age had a negative influence on snail response, but weight had none. Thus, snails crawled above the water until 15 days old in response to extract of the same-aged snails, and 3-day-old snails responded to the extract of any snails aged up to 60 days.

Responses of 3-Day-Old Snails to Egg and Adult Snails. The 3-day-old snails responded much less to the extracts of eggs, females, and males than to the extract of 3-day-old snails (Figure 2). The response was different among the extracts ($F_{5,30} = 29.809$, $P < 0.001$), but not among egg masses ($F_{5,30} = 1.305$, $P = 0.286$), and the interaction was significant ($F_{30,216} = 4.364$, $P < 0.001$). A Tukey's test revealed differences in response at $P < 0.05$ between the extracts of the 3-day-old snails and the others. Since responses to EDTA and the egg did not differ from each other, eggs did not cause a response.

Size-Dependent Response. Alarm responses of snails of all classes were highest at 0.5–1 hr (Figure 3). The response was different between extracts in all snail classes: $F_{4,624} = 55.308$, $P < 0.001$ in the 3-day-old snail; $F_{4,624} = 8.370$, $P < 0.001$ in the small snail; and $F_{4,624} = 14.685$, $P < 0.001$ in the medium snail. Responses were not different between large female and male snails ($0.046 \pm$

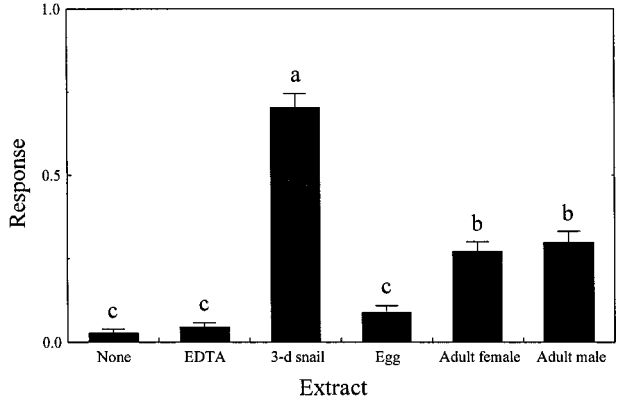


FIG. 2. Reponses of 3-day-old snails to 0.001 M EDTA and extracts of eggs, 3-day-old snails, adult females, and adult males. The response of the snails in the test tube without any extract is represented by "None." Columns and bars denote the mean and 1 SE, respectively. Letters above the bars indicate significant difference at $P < 0.05$ by a Tukey's test.

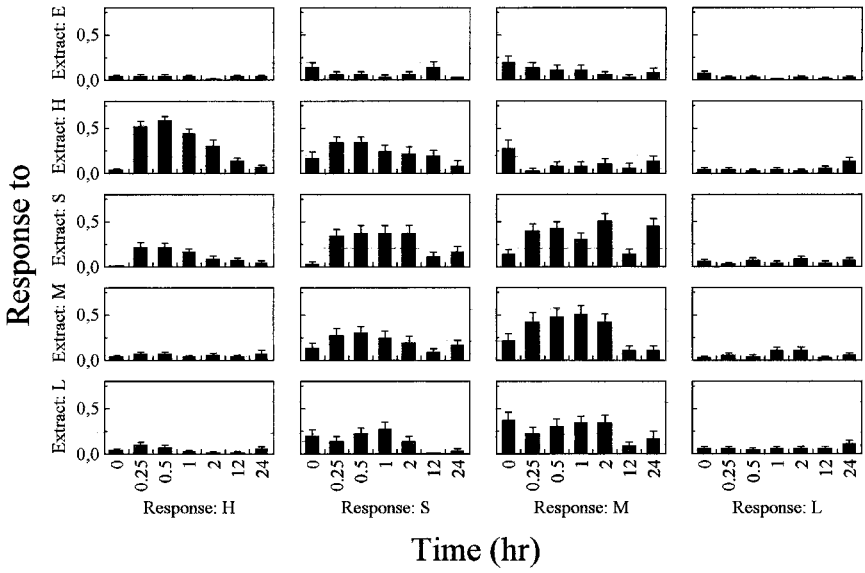


FIG. 3. Responses of 3-day-old (H), snail (S), medium (M) and large snails (L) to 0.001 M EDTA (E) and extracts of these snails. Bars correspond to observations made at 0.25, 0.5, 1, 2, 12, and 24 hr, and represent mean response ± 1 SE.

0.006 SE in female and 0.044 ± 0.006 SE in male; $F_{1,1077} = 0.108$, $P = 0.743$). Responses in each class snail were not different between the extracts of females and males ($F_{1,646} = 0.000$, $P = 0.998$), although the medium-sized snails responded more than the other snails did (Figure 4). Hence, the female and male large snails were pooled as the large class.

Since most responses occurred within 1 hr, snail responses within 1 hr were evaluated again by equation 2. Most 3-day-old snails crawled above the water in response to the extract of the same class and responded less or little to the other extracts (Table 1). On the other hand, most small and medium-sized snails buried themselves in soil in response to extract of the same or adjacent size classes. However, the large snails responded less to all extracts than the other snails did. The mean *Rp* shown by each class to the extract of the same class was different among classes (ANOVA, $F_{3,68} = 7.804$, $P < 0.001$). A Tukey's test showed a difference at $P < 0.05$ between the large class and the others, but the responses were not different among 3-day-old, small, and medium classes. Hence, small to medium snails contain cues stimulating alarm responses in other snails of their sizes, but large snails contain little or no cues to stimulate snails of any size.

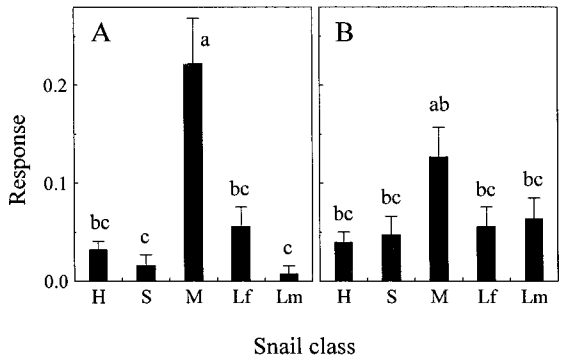


FIG. 4. Responses of 3-day-old (H), small (S), medium (M), large female (Lf) and large male (Lm) snails to extracts of large female (A) and large male (B) snails. Bars denote mean response ±1 SE with letters indicating significant difference at $P < 0.05$ by a Tukey's test.

TABLE 1. RESPONSE OF SNAILS OF EACH SIZE CLASS TO 0.001 M EDTA OR EXTRACT OF SNAILS OF EACH SIZE CLASS

Responding snails	Extract	Response (mean ± SD)			
		Crawling	Floating	Burying	Total
3-day-old	EDTA	0.00 ± 0.00	0.06 ± 0.08	0.02 ± 0.08	0.07 ± 0.12c
	3-day-old	0.61 ± 0.19	0.06 ± 0.12	0.00 ± 0.00	0.68 ± 0.12a
	Small	0.22 ± 0.23	0.09 ± 0.13	0.00 ± 0.00	0.32 ± 0.23b
	Medium	0.05 ± 0.11	0.07 ± 0.12	0.00 ± 0.00	0.12 ± 0.14c
	Large	0.04 ± 0.08	0.07 ± 0.12	0.02 ± 0.05	0.14 ± 0.16c
Small	EDTA	0.00 ± 0.00	0.03 ± 0.12	0.03 ± 0.12	0.06 ± 0.16c
	3-day-old	0.08 ± 0.19	0.00 ± 0.00	0.21 ± 0.31	0.31 ± 0.30b
	Small	0.00 ± 0.00	0.00 ± 0.00	0.44 ± 0.42	0.44 ± 0.42a
	Medium	0.03 ± 0.12	0.00 ± 0.00	0.44 ± 0.29	0.47 ± 0.27a
	Large	0.00 ± 0.00	0.03 ± 0.12	0.28 ± 0.26	0.31 ± 0.30b
Medium	EDTA	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.21	0.11 ± 0.21c
	3-day-old	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.21	0.11 ± 0.21c
	Small	0.00 ± 0.00	0.00 ± 0.00	0.47 ± 0.36	0.47 ± 0.36a
	Medium	0.00 ± 0.00	0.00 ± 0.00	0.61 ± 0.47	0.61 ± 0.47a
	Large	0.00 ± 0.00	0.00 ± 0.00	0.36 ± 0.38	0.36 ± 0.38b
Large	EDTA	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00b
	3-day-old	0.00 ± 0.00	0.03 ± 0.12	0.06 ± 0.16	0.08 ± 0.19ab
	Small	0.00 ± 0.00	0.00 ± 0.00	0.14 ± 0.29	0.14 ± 0.29ab
	Medium	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.35	0.25 ± 0.35a
	Large	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.19	0.08 ± 0.19ab

[a] Significant difference at $P < 0.05$ by a Tukey's test in the total response in each size class is represented by lowercase letters.

DISCUSSION

Alarm responses of age-varied snails to the extract of 3-day-old snails were age-dependent. Snails responded until 15 days old, but responses of older snails were much reduced. On the other hand, 3-day-old snails responded similarly to extracts of any snail between ages 1–60 days (Figure 1). Since shell lengths reach about 10 mm within one month after hatching (Estebenet and Cazaniga, 1992), the small snail in Table 1 corresponds to such age. Hence, snails change their alarm response from crawling above the water to self-burying at some age between 15 and 30 days. Furthermore, the alarm response can be elicited by an even more diluted extract than the extract used in the present study (Snyder and Snyder, 1971). Thus, the change in the alarm responses with the snail's growth can be explained by the predator–prey relationship, not by the way of making the extracts.

Since predators attack prey of sizes that are easy to handle (Cook, 1989), they adopt different attacking behavior in accord with prey sizes or select prey of limited sizes (Rowe, 1987; Alexander and Covich, 1991a). The predator's choice of prey size encourages prey to ignore attacks on different sizes, because of the cost of response (Slootweg, 1987; Rowe, 1987; Cook, 1989; Alexander and Covich, 1991b; Dodson et al., 1994; Chivers and Smith, 1998; Lewis, 2001). If different sized prey are attacked by different predators showing different behaviors, the prey will respond only when they perceive cues from the victims of similar sizes, and their responses may be size-dependent. If the hypothesis is true for *P. canaliculata*, predators should use different strategies to catch snails of different sizes.

Pomacea snails, which live in shallower places in rivers in Latin America (De Castellanos and Fernández, 1976), are preyed upon by aquatic insects and fingerlings when the snails are small, and by turtles, alligators, birds, and mammals when they are larger (Murphy, 1955; Vaz-Ferreira et al., 1965; Snyder and Snyder, 1971; Guimarães, 1981; Beltzer, 1985; Donnay and Beissinger, 1993; García, 1996; Santos et al., 1996; Stevens et al., 1999). Kai et al. (2001) report that carp of about 20 cm body length can eat snails with shells <10 mm. Although the carp is not native to Latin America, the results of Kai et al. suggest that fish of such a size preyed upon snails of these sizes there. Larger fish that can eat larger snails may not be able to approach the shore, but larger snails should be subject to predation by birds and mammals. In this situation, crawling above the water is safe for juvenile snails with shells <10 mm, and self-burial is efficient for larger snails.

Prey respond to signals from predators and victims (e.g., Crowl and Covich, 1990; Ball and Baker, 1996; Nyström and Åbjörnsson, 2000; Pettersson et al., 2000; Chivers et al., 2001; Chivers and Mirza, 2001; Laurila, 2001). If *P. canaliculata* snails respond to substances from the conspecifics, my results (Figures 1 and 2; Table 1) indicate that snails 1–15 days old contain substances that cause juvenile snails to respond, but older snails and eggs do not. This suggests that the substances

are produced when the snail body is formed and disappear at some age from 15 to 30 days. After the substances disappear, other cues that induce self-burying are produced in the snail. These new substances also disappear when the snails have shell lengths of about 30 mm. Thus, the snail's response to these substances changes with growth. In other words, the change in the snail's response to predators must be linked with the production of alarm substances in the body.

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ISOLATION OF BACTERIAL METABOLITES AS NATURAL INDUCERS FOR LARVAL SETTLEMENT IN THE MARINE POLYCHAETE *Hydroides elegans* (HASWELL)

TILMANN HARDER,[1] STANLEY CHUN KWAN LAU,[1] HANS-UWE DAHMS,[2] and PEI-YUAN QIAN[1,*]

[1]*Hong Kong University of Science and Technology*
Department of Biology
Clear Water Bay, Kowloon, SAR Hong Kong

[2]*Carl-von-Ossietzky Universität*
Fachbereich Biologie
D-26111 Oldenburg, Germany

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Abstract—The bacterial component of marine biofilms plays an important role in the induction of larval settlement in the polychaete *Hydroides elegans*. In this study, we provide experimental evidence that bacterial metabolites comprise the chemical signal for larval settlement. Bacteria were isolated from biofilms, purified and cultured according to standard procedures. Bacterial metabolites were isolated from spent culture broth by chloroform extraction as well as by closed-loop stripping and adsorption of volatile components on surface-modified silica gel. A pronounced biological activity was exclusively observed when concentrated metabolites were adsorbed on activated charcoal. Larvae did not respond to waterborne metabolites when prevented from contacting the bacterial film surface. These results indicate that an association of the chemical signal with a sorbent-like substratum may be an essential cofactor for the expression of biological activity. The functional role of bacterial exopolymers as an adsorptive matrix for larval settlement signals is discussed.

Key Words—*Hydroides elegans*, larval settlement, biofilm, chemical signal, bacteria, exopolymers.

* To whom correspondence should be addressed. E-mail: boqianpy@ust.hk

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INTRODUCTION

The life cycle of many sessile marine invertebrates includes a larval stage. A critical phase during the larval lifespan occurs when dispersed larvae gain the ability to attach and subsequently metamorphose into sedentary juveniles called settlement (Crisp, 1974; Scheltema, 1974; Chia, 1978). Larval settlement is a nonrandom process, i.e., surface exploration and the final choice of an attachment site are guided by specific signals associated with a marine substratum (Pawlik, 1992; Rodriguez et al., 1993).

The bioorganic film on marine surfaces is a major source of larval settlement signals (Brancato and Woollacott, 1982; Hadfield et al., 1994; Wieczorek and Todd, 1998; Maki, 1999). Biofilms are complex agglomerates of microorganisms (bacteria, diatoms, protozoa, and fungi) enclosed in a matrix of extracellular polymers (Costerton et al., 1995; Rittschof, 1997). The temporal and spatial variability of environmental factors such as tidal height, flow regime, and nutrient availability is distinctly reflected in the species richness and evenness of bacterial populations in marine biofilms (Strathmann and Branscomb, 1979; Strathmann et al., 1981; Wieczorek et al., 1995; Olivier et al., 2000). Due to their responsiveness to changes in environmental conditions, bacteria are important informative signposts for surface-exploring larvae. A broad range of marine invertebrate larvae are able to distinguish between bacterial communities of different composition, physiological condition, and growth phase, indicating a subtle and finely tuned larval response to bacteria-derived information about the substratum (Bonar et al., 1986; Le Tourneux and Bourget, 1988; Pearce and Scheibling, 1991; Keough and Raimondi, 1996).

The induction of larval settlement by bacteria is reportedly due to both waterborne metabolites (Cameron and Hinegardner, 1974; Fitt et al., 1990; Rodriguez and Epifanio, 2000) and cell surface-associated signals (Kirchman et al., 1982; Maki and Mitchell, 1985; Maki et al., 1990; Szewzyk et al., 1991). Only a few classes of bacteria-derived settlement signals have been recognized, such as lipids (Leitz and Wagner, 1993; Schmahl, 1985), oligopeptides (Neumann, 1979), glycoconjugates (Kirchmann et al., 1982; Szewzyk et al., 1991), and DOPA-mimetics (Weiner et al., 1989). However, no larval settlement signal of bacterial origin has been purified or elucidated so far. The underlying mechanism of the induction of larval settlement by marine bacteria is largely unknown.

In this study, we extended our previous investigations on the induction of larval settlement in the polychaete *Hydroides elegans* (Haswell) by marine bacteria. The assessment of individual bacterial isolates revealed an equiproportional ratio of highly inductive to moderately inductive to noninductive bacterial species (Lau and Qian, 1997; Unabia and Hadfield, 1999; Lau et al., 2002). Larval settlement was exclusively invoked by viable cells of inductive bacteria (Lau and Qian, 2001). To investigate the role of bacterial metabolites as presumptive chemical signals

for larval settlement in *H. elegans*, we intended a comprehensive bioassay-guided isolation and gas-chromatographic analysis of bacterial metabolites. This investigation included the metabolites of four bacteria: *Roseobacter* sp., *Rhodovulum* sp., *Micrococcus* sp., and *Vibrio* sp. The former two are inductive; the latter two are noninductive for larval settlement in *H. elegans* and served as control strains.

METHODS AND MATERIALS

Larval Culture Procedures. Colonies of *Hydroides elegans* were obtained from submerged rafts at a fish farm in Clear Water Bay, Hong Kong. The tubes of sexually mature individuals were cracked to release gametes. After fertilization, gametes were transferred into aerated 2-liter beakers containing 0.22-μm-filtered seawater (FSW) and algal food medium (*Isochrysis galbana*, Tahitian strain, 10^5 cells/ml). The larval culture was incubated at 24°C under a 15:9 hr (light:dark) photocycle. After 4 days, larvae were transferred into new FSW and food medium. When the morphology indicated competence (Wisely, 1958) after day 5 or 6, the larvae were sieved out and immediately used in bioassays.

Bacterial Cultures. Stock cultures of bacteria (*Roseobacter* sp., *Rhodovulum* sp., *Micrococcus* sp., and *Vibrio* sp.) were obtained from the Marine Bacteria Culture Collection at the Hong Kong University of Science and Technology (strain designations UST950701-009, UST950701-012, UST950701-006, and UST950701-007, respectively). Aliquots (2 ml) of stocks were individually inoculated into 300 ml of sterile culture broth [0.5 % (w/v) peptone, 0.3 % (w/v) yeast extract] prepared with charcoal-filtered artificial seawater (ASW). These cultures were incubated at 30°C and grown for 48 hr to stationary phase in order to enrich the broth with bacterial metabolites.

Liquid–Liquid Extraction of Bacterial Metabolites. Spent culture broth was separated from bacterial cells by centrifugation and sterile filtration (0.22 μm). Bacterial metabolites were isolated from broth by liquid–liquid extraction (LLE) in disposable hydromatrix-filled cartridges (Chem-Elut, 5 ml; Varian Inc). Cartridges were loaded with 5 ml of broth and extracted with 5 ml of chloroform (analytical grade). Extracts were concentrated to 1 ml under *vacuo* and stored at −80°C until analysis. Autoclaved culture broth and ASW were treated accordingly and served as controls. Extractions were repeated twice with samples from different batches of bacterial cultures.

Isolation of Volatile Bacterial Metabolites. Volatile bacterial metabolites were isolated by closed-loop stripping (CLS) according to Grob and Zürcher (1976). We modified the CLS apparatus to serve as a microbial bioreactor (Figure 1). For this purpose, a 2-liter vessel (Normag) was filled with 1.5 liters of culture broth, autoclaved, and subsequently inoculated with bacteria. The closed loop was formed by connecting the vessel with a 0.22-μm membrane-filter (Microfiltration Systems) and two cartridges of preconditioned, surface-modified silica gel (C_8 and

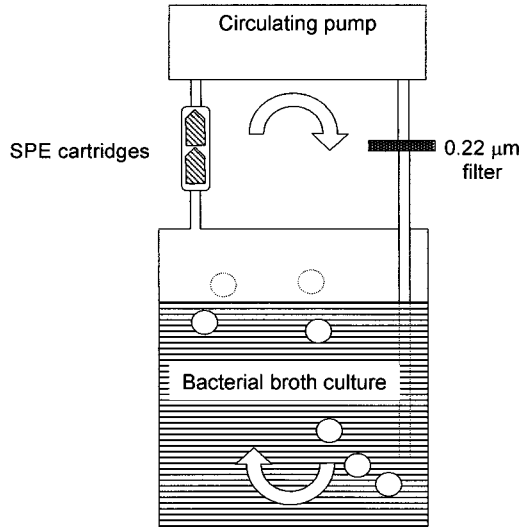


FIG. 1. Schematic diagram of the closed-loop stripping apparatus. The bacterial culture broth was continuously purged by a circulating air stream. Volatile metabolites were adsorbed on pre-conditioned, surface-modified silica gel (C_8 and CN).

CN; 50 mg, Bond-Elut, Varian Inc.). The utilization of solid-phase extraction cartridges for CLS has been reported previously (Priesnitz, 2000). The headspace volume of approximately 500 ml was continuously circulated (ca. 30 ml/min) through the system by a magnetically clutched micropump (Metal Bellows Corp.). Besides sweeping volatile metabolites out of the aqueous phase onto the sorbents, this principle served to aerate the bacterial culture, keep the sorbents moist, and sterilize the circulating airstream. The bacterial culture was grown in the vessel at 25°C for 48 hr while volatile metabolites were stripped and trapped. Subsequently, the cartridges were eluted with 50 μl of acetone (analytical grade). Controls were prepared by stripping culture broth without bacteria. The isolation procedures were repeated twice with samples from different bacterial cultures. After CLS, aliquots of cultures were grown on marine agar to check for potential contamination by other bacteria.

In addition to CLS, a purge-and-trap system (Tekmar) was used to isolate volatile metabolites from stationary phase cultures. This procedure served as a qualitative control to evaluate the efficiency of the preparative CLS methodology. A small volume (50 ml) of spent culture broth was purged with helium for 15 min, while volatile components were trapped on a Carbowax cartridge (Tekmar: ALS 2016; LSC 2000). Adsorbed compounds were subsequently concentrated in a liquid-nitrogen-operated cryofocusing module after heating the Carbowax cartridge to 150°C. Autoclaved culture broth and ASW were analyzed accordingly

and served as controls. The isolation procedures were repeated twice with samples from different batches of cultures.

Analytical Procedures. All samples were analyzed by coupled gas chromatography–mass spectrometry (GC-MS). Samples were separated by GC (Hewlett-Packard 5890 Series II) on a fused silica capillary column (DB5; 30 m; 0.25 mm ID). The temperature program was as follows: 5 min isotherm at 40°C, 40–150°C at 10°C/min, and 2 min isotherm at 150°C. Samples were injected on-column (1 μl) or, in the case of purge-and-trap, by heating the cryo-focusing module to 200°C. Samples were detected by MS (HP 5972) at 70 eV. The ion source temperature was 162°C; the scanning range was m/e 40–300.

Larval Bioassays with Bacterial Films. Bacteria at stationary growth phase were harvested from broth cultures, washed by centrifugation (3000*g*), and resuspended in sterile FSW at 10^8 cells/ml. Aliquots (5 ml) were transferred into polystyrene Petri dishes (Falcon, #1006) and incubated for 3 hr for the attachment of bacteria on the dish surface. Subsequently, the dishes were dip-rinsed (10 times) and filled with sterile FSW and 20 larvae. This experimental setup is referred to as the "single-dish assay." A double-compartment test vessel was used to concurrently test the effect of attached bacteria and their waterborne metabolites on larval settlement (double-dish assay). The compartment, one of which was covered with a bacterial film, were separated by a nonpenetrable mesh for larvae (90 μm). The vessel received 40 larvae (20 per compartment) and was mounted on an orbital shaker (50 rpm) to facilitate the diffusion of waterborne bacterial metabolites during the bioassay.

Larval Bioassays with Isolated Bacterial Metabolites. Aliquots (50 μl) of bacterial metabolites in the chloroform and acetone extracts were adsorbed onto charcoal wafers (self-made from carbonized wood) and glass slides (Corning). The charcoal wafers ($5 \times 5 \times 1$ mm) were activated by heat (200°C, 2 hr), and glass slides were acid-rinsed prior to utilization. Control substrata were prepared with pure organic solvents. Pretreated substrata were fixed on the bottom of the wells of a multiwell tissue culture plate (Falcon, #3043) with double-sided adhesive tape. Individual wells were filled with 2 ml of FSW and 20 competent larvae.

Assessment of Biological Activity. In all bioassays, larval behavior was continuously monitored throughout the first 30 min and subsequently recorded after 1, 2, 4, and 24 hr. Each sample (five replicates) was investigated along with controls of unfilmed Petri dishes or solvent-treated charcoal and glass substrata. The settlement stage of the larvae was determined according to morphological descriptions in Carpizo-Ituarte and Hadfield (1998). Experiments were repeated twice with different batches.

Statistical Analysis. All bioassays were performed with five replicates. The percentages of larval settlement in individual experimental treatments were arcsine-transformed. In order to improve the transformation, a value of $1/(4x)$ (x representing the number of larvae per test vessel) was assigned to replicates with no

larval settlement (Zar, 1996). Since settlement data did not meet the normality assumption (Shapiro-Wilk's W test), they were analyzed nonparametrically. This was achieved by transformation of values to ranks followed by one-way ANOVA and Tukey's multiple comparison (Conover and Iman, 1981). Pairwise comparisons were performed with Student's *t* test. The data presented in the figures are not transformed. Results were considered reproducible when individual statistical evaluations of experimental repetitions produced the same statistical outcome.

RESULTS

Larval Response to Bacterial Films. In the single-dish assay, larvae immediately displayed a distinctive settlement response upon encountering films of *Roseobacter* sp. and *Rhodovulum* sp., i.e., they established surface contact and began to crawl over the film surface. Within 5 min, larvae firmly attached and began to secrete the primary mucous tube. During the next 4 hr, attached larvae developed branchial lobes, which subsequently developed into radioles. After 5 hr, juvenile worms secreted a calcareous tube. No statistical difference was observed between the larval response to films of *Roseobacter* sp. and *Rhodovulum* sp. ($p = 0.668$; 24 hr). In contrast, the percentage of responding larvae was lower in treatments with bacterial films of *Micrococcus* sp. and *Vibrio* sp. ($p < 0.001$; 24 hr) (Figure 2A). In the double-dish assay, the larval response to films of *Roseobacter* sp. and *Rhodovulum* sp. was analogous to the single-dish assay above, whereas no response was observed in the respective unfilmed compartment of the test vessel (Table 1). The bacterial film density in these assays was in the range of 10^5 to 10^6 cells/mm^2.

Larval Response to Bacterial Metabolites. The chloroform extracts induced significant levels of larval settlement when adsorbed to charcoal. Settlement occurred exclusively on the surface of the charcoal wafer, but not on the test vessel. No statistical difference in larval settlement was observed among the four metabolite-containing samples ($p > 0.153$, Tukey's test; 24 hr; Figure 2B). No settlement was observed in the negative controls (i.e., charcoal pretreated with pure

TABLE 1. PERCENTAGE OF LARVAL SETTLEMENT IN CONCURRENT INVESTIGATION OF BACTERIAL FILMS AND BACTERIAL METABOLITES (DOUBLE-DISH ASSAY)[a]

	Compartment		
	Filmed	Unfilmed	*t* test
Roseobacter sp.	44 (±13.87)	0	$p < 0.01$
Rhodovulum sp.	40 (±20)	1 (±2.24)	$p < 0.01$

[a] Data are mean percentages (±1 SD; 5 replicates) of larval settlement in the filmed and unfilmed dish, and on the nylon mesh after 5 h.

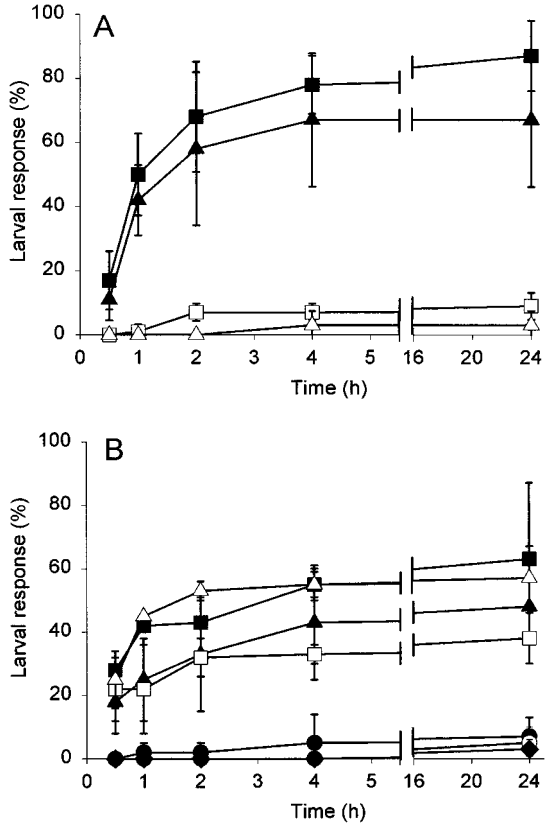


FIG. 2. (A) Larval settlement on monospecific films of four bacterial strains, and (B) larval settlement on charcoal-adsorbed chloroform extracts of bacterial metabolites and three controls, i.e., charcoal pretreated with pure chloroform (●), and chloroform extracts of autoclaved artificial seawater (○) and bacterial culture broth (◆). Data are mean percentages (±1 SD; 5 replicates) of larval settlement at different observation times. ■ = *Roseobacter* sp.; ▲ = *Rhodovulum* sp.; □ = *Micrococcus* sp.; △ = *Vibrio* sp.

chloroform, and chloroform extracts of sterile ASW and culture broth) ($p < 0.04$, 24 hr; Figure 2B, Table 2). With the exception of *Rhodovulum* sp. ($p = 0.093$, *t* test; 24 hr; Figure 3), the percentage of settlement on glass was lower than on charcoal ($p < 0.016$, 24 hr; Figure 3). The volatile metabolite samples originating from the CLS procedure did not induce larval settlement (Table 2).

Analytical Results. The analysis of major constituents in the metabolite samples originating from the different isolation procedures and bacterial strains is summarized in Table 2. The major composition differed with respect to the isolation

TABLE 2. COMPOSITION AND BIOLOGICAL ACTIVITY OF METABOLITE-CONTAINING SAMPLES FROM THREE ISOLATION PROCEDURES AND FOUR BACTERIAL SPECIES UNDER INVESTIGATION[a]

	Closed-loop stripping		Purge-and-trap		Liquid-liquid extraction	
Bacterium	BA	Major components	BA	Major components	BA	Major components
Roseobacter sp.	(−)	Alkanes (C_5–C_{12}) Alkenes (C_6–C_{10}) Alkylated hydroxy ketones (C_5–C_6) Aromatic nitrile Alkylated phenol Butanoic acid ester^{CN}	na	Dimethylated or organosulfur compounds (di- and trisulfides)	(+)	R_t = 3–11 min (see Fig. 4) Mono- and diketones (C_5–C_9) Hydroxylated ketones (C_5–C_7) Aromatic alcohols Alkylated alkanes (C_7–C_{16}) Alkenes (C_8–C_{10}) Alkylated butanoic acid ester
Vibrio sp.	(−)	Acetic acid Alkenes (C_{12}–C_{14}) Alkylated hydroxy ketones (C_5–C_6) Alkylated phenol	na	Dimethylated or organosulfur compounds (di- and trisulfides)	(+)	R_t = 11–13 min Alkylated alkanes (C_{12}–C_{18}) Alkenes (C_{10}–C_{12})
Rhodovulum sp.	(−)	Alkanes (C_7–C_{12}) Alkanes (C_{12}–C_{14}) Alkylated hydroxy ketones (C_5–C_6) Alkylated phenol	na	Dimethylated or organosulfur compounds (mono-, di- and trisulfides) Butylated hydroxytoluene	(+)	R_t = 13–17 min Alkylated alkanes (C_{18}–C_{20})
Micrococcus sp.	(−)	Alkanes (C_6–C_{12}) Alkanes (C_6–C_{10}) Alkylated hydroxy ketones (C_5–C_6) Butanoic acid ester^{CN}	na	Dimethylated or organosulfur compounds (mono-, di- and trisulfides)	(+)	

Note: ^{CN} refers to compounds retained on the silica-bonded nitrile phase (CN). If not indicated otherwise, compounds were retained on octyl phase (C_8).

[a] The biological activity (BA) is denoted by the symbols (+) and (−). The composition of bacterial metabolites from closed-loop stripping and purge-and-trap is given for each bacterial strain. The composition of the liquid–liquid extracted metabolites of the four strains is summarized in the last column.

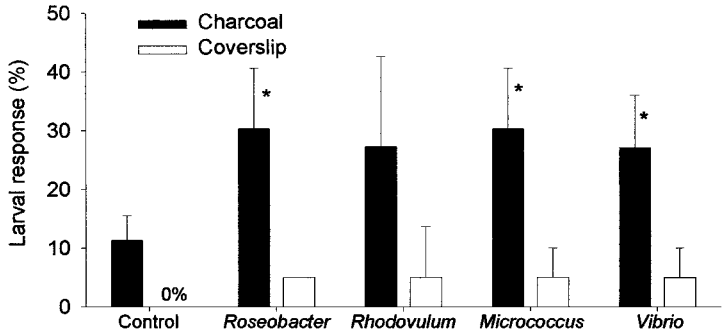


FIG. 3. Larval settlement in response to charcoal- and glass-adsorbed chloroform extracts of bacterial metabolites. Data are mean percentages (±1 SD; 5 replicates) of larval settlement after 12 h. * Statistically significant difference (Student's t test, $\alpha = 0.05$).

procedure but was relatively homogenous among the four different bacterial strains. The metabolites from CLS consisted mainly of alkanes, alkenes, and alkylated hydroxyketones, while dimethylated oligosulfides comprised the major compound class in the purge-and-trap isolation. The composition of the chloroform extracts was more complex compared to that from CLS but comprised the same classes of compounds. The gas chromatograms of the chloroform extracts revealed a similar peak pattern among the four bacterial strains (Figure 4). The broad assignment of compound classes is summarized for the retention windows of 3–11, 11–13, and 13–17 min (Table 2).

DISCUSSION

The aim of this study was to isolate bacterial metabolites and investigate their presumptive role as chemical signals for larval settlement in *Hydroides elegans*. Bacterial suspension cultures were used as sources for bacterial metabolites. This approach was advantageous over the production of metabolite-enriched seawater from bacterial films in order to yield bacterial metabolites in a preparative scale. The bacteria chosen belong to different phylogenetic branches, including gram-positive (*Micrococcus* sp.), *α-Proteobacteria* (*Roseobacter* sp. and *Rhodovulum* sp.), and *γ-Proteobacteria* (*Vibrio* sp.).

A broad isolation strategy for bacterial metabolites, encompassing liquid–liquid extraction and closed-loop stripping, was chosen for the enrichment of the putative chemical signals for larval settlement. A general description of the CLS technique and detailed reports of successful isolations of volatile pheromones from seawater samples can be found in Zeeck et al. (1991), Gassmann et al. (1995), and Bartels-Hardege et al. (1996). The two isolation procedures resulted in chloroform

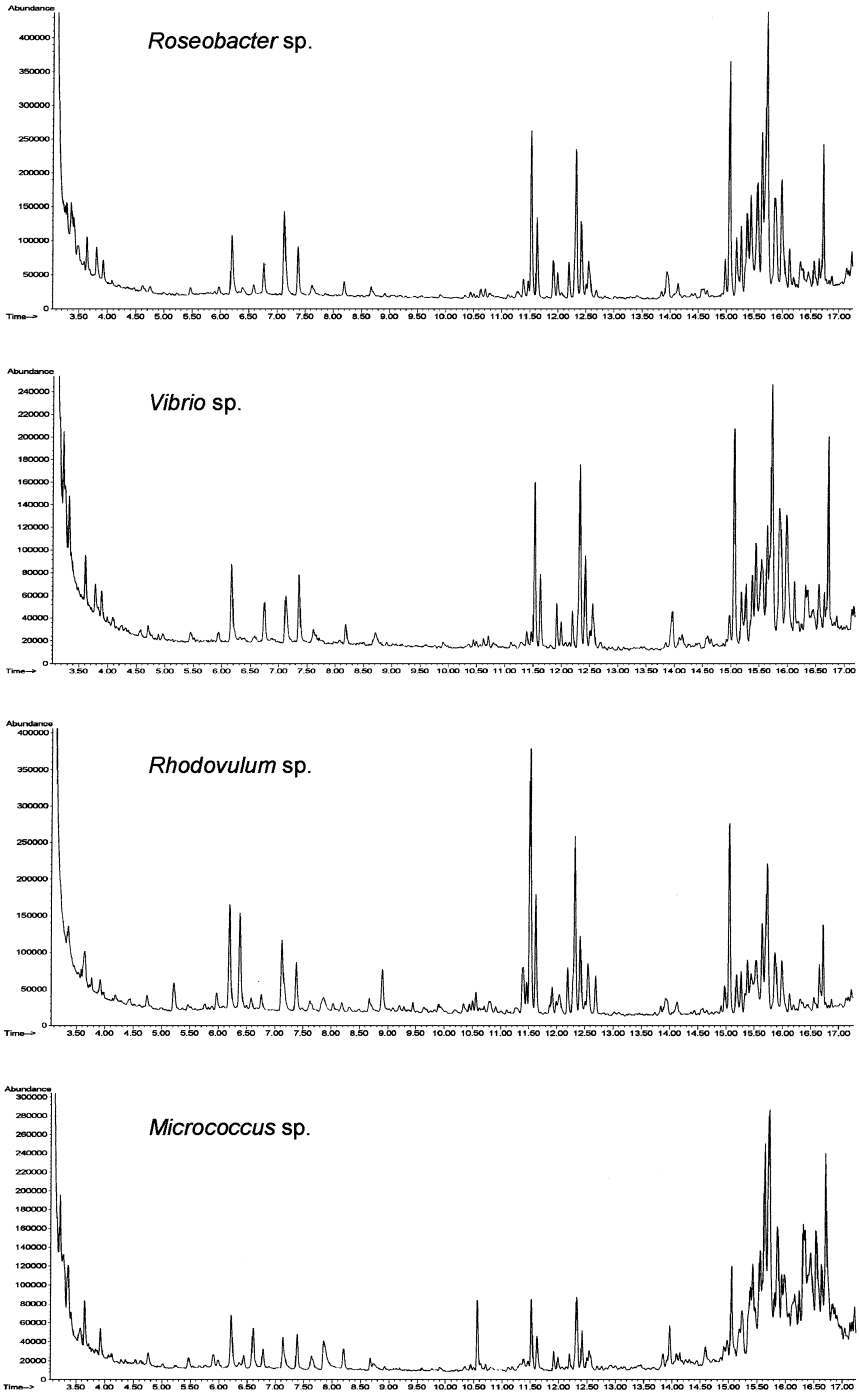

Abundance
Roseobacter sp.
Time-->
Abundance
Vibrio sp.
Time-->
Abundance
Rhodovulum sp.
Time-->
Abundance
Micrococcus sp.
Time-->

FIG. 4.

extracts containing mainly lipophilic metabolites and acetone extracts containing volatile metabolites in a wide range of polarities. In order to remove the organic solvents, the samples were adsorbed on activated charcoal and glass substrata, which served as vehicles for bacterial metabolites in larval bioassays.

The larval response to the charcoal-adsorbed chloroform extracts was the same as that to the inductive films of *Roseobacter* sp. and *Rhodovulum* sp., both in terms of the magnitude of the larval response (Figure 2A) and the rate of morphogenesis (see Carpizo-Ituarte and Hadfield, 1998, for morphogenetic details). Since the controls did not elicit any larval response in the bioassay, the observed biological activity was likely due to extracted bacterial metabolites rather than culture broth components. This result shows for the first time that a fraction of waterborne bacterial metabolites served as a chemical signal for the induction of larval settlement in *H. elegans*. It further indicates that larval settlement is not due to a synergistic effect of dissolved and surface-bound signals. This possibility has been raised previously by Unabia and Hadfield (1999) and Lau and Qian (2001), and has been confirmed in the oyster *Crassostrea gigas* (Bonar et al., 1986; Fitt et al., 1990).

In contrast to the pronounced biological activity of the charcoal-adsorbed chloroform extracts, no larval response resulted from air-dried extracts on glass slides. The latter is likely due to the absence of biologically active components on the glass slide, suggesting that the association of the chemical signal with a suitable substratum is an essential cofactor for the induction of larval settlement by bacterial metabolites. A similar concept was supported by the outcome of the concurrent investigation of bacterial cells and waterborne metabolites in the double-dish assay (Table 1). Here, the bacterial metabolites originated exclusively from the compartment bearing the bacterial film. However, no concentration gradient of waterborne bacterial products was supposed to be present in the agitated vessel, as indicated by the rapid and homogenous diffusion of a dye in a control vessel (data not shown). Since larval settlement was exclusively observed in the filmed compartment, the presence of the bacterial film was thought to be essential for the inductive effect of bacterial metabolites. The combined observations of induction of larval settlement by waterborne bacterial metabolites and the exclusive occurrence of larval settlement in the filmed compartment of the test vessel indicated that an association of waterborne metabolites with the bacterial film surface was prerequisite for the inductive effect.

We hypothesize that the association of bacterial metabolites with the film surface is facilitated by high binding affinities of exopolymers surrounding

←

FIG. 4. Gas chromatograms of chloroform extracts of bacterial metabolites of *Roseobacter* sp., *Vibrio* sp., *Rhodovulum* sp., and *Micrococcus* sp. GC conditions (Hewlett-Packard 5890 Series II): 30 m fused silica capillary column (DB-5), 0.25 mm ID; temperature program: 40°C for 5 min, 40–150°C at 10°C/min, 150°C for 2 min.

the bacterial cells. Exopolymers are surface-active molecules comprised of heteropolysaccharides, proteins, and glycoproteins. They can be regarded not only as an adsorptive "sponge," but also as a diffusion barrier for metabolites (Decho, 1990). We have previously demonstrated that exopolymers themselves do not evoke a settlement response in *H. elegans* larvae (Lau et al., unpublished). However, the results of the current study indicate exopolymers are essential as mediators of larval settlement or as vehicles for chemical signals. Owing to their distinctive binding properties (Rees, 1976), exopolymers may retain bacterial metabolites on the cell surface and, thus, render the induction of larval settlement. This hypothesis is in accord with a similar suggestion on the role of bacterial exopolymers in mediating larval settlement in oysters (Decho and Zimmer-Faust, 1996).

Thus far, our data demonstrated that the four bacterial strains produced a chemical signal for larval settlement in suspension culture. However, this effect was not paralleled by the induction activity of these strains' monospecific films. In contrast to *Roseobacter* sp. and *Rhodovulum* sp., the monospecific films of *Micrococcus* sp. and *Vibrio* sp. did not evoke larval settlement (Figure 2A). What was the reason for this discrepancy? Provided that exopolymers mediate larval settlement by binding inductive bacterial metabolites to the cell surface, the lack of a settlement response on the films of *Micrococcus* sp. and *Vibrio* sp. may be related to the properties of exopolymers in these bacteria. It is well known that the quantity and quality of exopolymers may vary drastically among strains or even within the same strain under different conditions (Sutherland, 1985; Decho, 1990). Moreover, bacteria lacking exopolymers are common (Cowen, 1992). Thus, the effect of a monospecific bacterial film on larval settlement may likely be affected by the possession, quantity, and quality of exopolymers. However, a correlation of the bacterial strains activity on larval settlement with the properties of their exopolymers is currently not possible since the relative amounts of exopolymers of the four strains under investigation, as well as their composition, are unknown. An alternative explanation for the discrepancy may be that *Micrococcus* sp. and *Vibrio* sp. produce lower quantities of inductive metabolites *Roseobacter* sp. and *Rhodovulum* sp. Provided this case, biological activity was detected only after enriching the metabolites by the isolation procedures employed in this study.

The chromatographic and spectroscopic analysis of isolated metabolites revealed a similar composition among the four bacteria (Figure 4, Table 2). The bulk of components in the chloroform extracts (LLE) was dominated by compounds of low to medium polarity, such as alkylated alkanes, alkenes, and alkylated hydroxyketones in the volatility range bracketed by *n*-heptane and *n*-eicosan. The composition of the more volatile fraction of bacterial metabolites enriched by the CLS technique differed more distinctly among the individual bacterial strains, but the major compound classes were the same as in the chloroform extracts. However, these samples did not evoke a larval response in the bioassay. The purge-and-trap analysis specifically pointed to high concentrations of dimethylated oligosulfides

in the volatile fraction of bacterial metabolites. These compounds are well known as bacterial degradation products of organic sulfur compounds (McCarty et al., 1993). Since the sulfides were absent in the samples obtained by LLE, they may not contribute to the induction activity.

We are currently developing a liquid-chromatographic protocol for a preparative bioassay-guided isolation and purification of the biologically active chloroform extracts. The similarity in composition of the four extracts under investigation may allow the separation procedure to be standardized.

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INFLUENCE OF *Myriophyllum spicatum*-DERIVED TANNINS ON GUT MICROBIOTA OF ITS HERBIVORE *Acentria ephemerella*

OLIVER WALENCIAK,[1] WALTER ZWISLER,[2] and ELISABETH M. GROSS[1,*]

[1]*Limnological Institute*
University of Konstanz
78457 Konstanz, Germany

[2]*Qualis Laboratory*
Blarerstr. 56
78462 Konstanz, Germany

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Abstract—The submerged living larvae of *Acentria ephemerella* were fed in the laboratory with either *M. spicatum* or *Potamogeton perfoliatus*, two of their host plants. Larvae exhibited a reduced growth when fed *M. spicatum*, a freshwater angiosperm that contains high concentrations of tannins, secondary metabolites known for their herbivore-deterrent and antimicrobial properties. In this study, we investigated the influence of food-derived tannins on gut microbiota. Bacterial densities in the guts did not differ between the food regimes, ranging from 2.8 to 13.3 $\times$ 10^6 cells per gut. Gut bacteria were characterized with cultivation techniques and subsequent identification of the strains by molecular methods. We isolated 17 bacterial strains belonging to all subdivisions, i.e., we identified α-, β-, and γ-proteobacteria, *Cytophyaga/Flavobacteria* (CF) and several Gram-positive bacteria. All except one Gram-positive strain were found in the guts of larvae fed with *P. perfoliatus*. Gram-positive bacteria and bacteria of the CF cluster were more sensitive to polyphenol-containing extracts of *M. spicatum* in an agar diffusion assay than strains of the α- or γ-proteobacteria subdivision. Our results suggest an influence of food-derived tannins on gut microbiota in *A. ephemerella*.

Key Words—Aquatic angiosperm, gut symbionts, hydrolyzable tannin, polyphenol, freshwater macrophytes.

* To whom correspondence should be addressed. E-mail: Elisabeth.Gross@uni-konstanz.de

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INTRODUCTION

Tannins (polyphenols) are secondary metabolites known for their ability to bind and precipitate proteins or enzymes (Spencer et al., 1988). However, some herbivores are well adapted to tannin-containing plants (Manuwoto and Scriber, 1986; Barbehenn and Martin, 1992). Despite numerous investigations, the precise mechanisms of tannin–herbivore interactions are still unknown.

An important function of tannins in plants is to provide protection against a wide range of potential phytopathogenic microorganisms (Field and Lettinga, 1992). Tannins possess diverse antimicrobial activities (Scalbert, 1991); thus, tannins could also be influencing gut microbiota of herbivores. Consequently, Schultz et al. (1992) hypothesized "that any negative impacts of plant polyphenols on insects or vertebrates may be no more than a fortuitous side effect of plant interactions with microbes."

Larvae of the aquatic moth *Acentria ephemerella* (Lepidoptera: Pyralidae) feed on both tannin-containing and tannin-free freshwater macrophytes, causing substantial damage (Gross et al., 2001, 2002). *Acentria* grows significantly more slowly when fed *Myriophyllum spicatum* than when fed tannin-free pondweeds (Choi et al., 2002). *M. spicatum* contains approximately 10% tannins based on dry weight (Gross et al., 1996).

The impact of food-derived tannins on gut microbiota of lepidopteran larvae has been investigated only in the economically important species *Lymantria dispar* and its pathogen *Bacillus thuringiensis* (Appel and Schultz, 1994). The present study was conducted to characterize naturally occurring gut bacteria of the freshwater moth *Acentria ephemerella.* We compared the gut microbiota of larvae fed tannin-free *P. perfoliatus* and larvae fed tannin-containing *M. spicatum* to each other and to those epiphytic bacteria found on the respective host plants. Finally, we investigated the susceptibility of gut microbiota to extracts from both macrophytes and pure phenolic compounds.

METHODS AND MATERIALS

Sampling. M. spicatum and *Potamogeton perfoliatus*, both food plants of *Acentria* larvae (Gross et al., 2002), were sampled either by snorkeling or by SCUBA diving in Untersee, a shallow part of Lake Constance, Germany, in August 2001. Samples were stored in coolers and transferred to a 4°C cooling chamber at the institute. The fresh plant material was divided into three groups the next day. Apical shoot sections (upper 2–5 cm of shoots) and green leaves from the upper 25 cm were separated from the first group of plants. These were ground with a mortar and pestle in liquid nitrogen and freeze-dried separately for further

extraction. Individual leaves were picked for harvesting epiphytic bacteria from the second group. Leaves were chosen randomly from the upper 25 cm; no apical shoot sections were taken. Larvae were picked from the remaining plant material and kept in 1.5-liter glass jars in lake water. Larvae were only collected from *P. perfoliatus* since no larvae were present on *M. spicatum* at the sampling date. Larvae were divided into two groups and, for two days, fed constantly with apical shoot sections of either *M. spicatum* or *P. perfoliatus*.

Harvesting Bacteria. To isolate epiphytic bacteria, about 10 mg fresh weight (FW) of leaf material was suspended in 1 ml sterile sodium tetrapyrophosphate (PPi) solution (0.1 M) in an Eppendorf vial (1.5 ml). PPi had been shown to facilitate a good recovery of attached bacteria in arthropod guts (Cazemier et al., 1997). Preliminary experiments revealed PPi also as an appropriate detergent for harvesting epiphytic bacteria, since it did not show any influence on growth of isolated bacteria. The leaves were sonicated in an ultrasonic bath for 30 sec, shaken for 15 min at 1100 rpm in an Eppendorf thermomixer, and then sonicated for another 30 sec. One aliquot of this bacterial suspension was taken for direct counts of bacteria; the remaining suspension was used for cultivation.

In order to retrieve gut bacteria, larvae were first sterilized with 70% ethanol to avoid contamination with microorganisms attached to their surface. Larvae were dissected under a stereomicroscope; the complete intestinal tract was removed and separated into fore-, mid-, and hindgut. Each gut segment was separately immersed in 250 μl sterile PPi, squeezed with a sterile preparation needle, and treated by sonication as described above to yield gut bacterial suspensions.

Bacterial Counts. One hundred microliters of bacterial suspensions, either from leaves or *Acentria* guts, were fixed with 100 μl formaldehyde (10%, v/v) and diluted with water to a final volume of 1 ml. We used only ultrapure and sterilized water for microbiological and biochemical analysis. Staining was performed with 100 μl of 4′,6-diamidino-2-phenylindole (DAPI; 10 μg/ml in 1% formaldehyde, v/v), and the mixture was incubated for 30 min in the dark. The stained bacteria were filtered on 0.2 μm black Nucleopore filters and counted under a Nikon epifluorescence microscope (Labophot 2). Of each microscopic preparation, 10 microscopic fields (0.08 $\times$ 0.08 mm) were counted.

The surface area of the freshwater macrophytes used was calculated from the dry weight of the plant samples according to Sher-Kaul et al. (1995). Bacterial densities in the gut of *Acentria* were quantified per gut segment (fore-, mid-, or hindgut) and per volume of gut fluid. For the latter we had to make some approximations. Although we used third to fourth instar larvae (for size classes see Gross et al., 2001, 2002), they were still too small to determine their gut volumes accurately. Consequently, we measured the head capsule widths to determine larval size. For an estimate of the gut volume, we assumed that the length of the larvae was correlated with the head capsule width and converted the latter to length

sizes (100 μm head capsule width set equivalent to 1.6 mm length). We further estimated a mean gut diameter of 1 mm and calculated gut volumes using these measures.

Isolation of Bacteria. Bacterial suspension in appropriate dilutions was spread on modified CPS agar (Collins and Willoughby, 1962) containing 0.5 g tryptone (Oxoid), 0.5 g peptone (Difco), 0.5 g soluble starch (Merck), 1 ml glycerol, 0.2 g K_2HPO_4, and 0.05 g $MgSO_4$. The plates were incubated aerobically for 3 days at 24°C. This method yielded distinct colonies. Representative colonies were selected on the basis of morphological criteria, such as colony size, pigmentation or surface structure, and recultured. Several isolates were obtained from guts and leaves, some from different sources looked similar. Therefore, all isolates were compared by means of denaturing gradient gel-electrophoresis (DGGE) for further characterization.

PCR and DGGE. Bacterial DNA of cultured cells was isolated in water at 100°C for 15 min. After centrifugation for 5 min at 13,600 g, 1 μl of supernatant containing bacterial DNA was taken as template for PCR. Small subunit rRNA genes were amplified by PCR using the primers 5′-GC-clamp-CCTACGGGAGGC AGCAG-3′ (forward) and 5′-CCGTCAATTCMTTTGAGTTT-3′ (reverse) (Muyzer et al., 1998). PCR was performed in 50-μl assays with a GENIUS thermocycler (Techne, Cambridge, UK). One unit of Taq DNA polymerase (Eppendorf Master Taq) was used. PCR conditions were an initial denaturation step of 3 min at 94°C, followed by a touchdown protocol of 20 cycles in which the DNA was denatured for 1 min at 94°C, annealed for 1 min starting at 65°C, and decreased 0.5°C/cycle to 55°C and extended for 2 min at 72°C. This was followed by nine cycles in which the annealing temperature was 55°C. PCR products were examined using agarose gel electrophoresis and ethidium bromide staining.

DGGE of PCR products was performed by the method described by Muyzer et al. (1996) with the use of a DCode System (Bio-Rad Laboratories). Products were analyzed on a 6% (w/v) polyacrylamide gel with a linear gradient from 20 to 60% denaturant [100% denaturant contained 7 M urea and 40% (v/v) formamide]. Electrophoresis was performed for 16 hr at 95 V and a temperature of 60°C. After electrophoresis, gels were stained for 15 min with SYBR Gold (0.1 μl/ml; Molecular Probes), rinsed in water, and photographed on a UV transilluminator with a black and white Polaroid camera. Isolates with different banding patterns were taken as different bacterial species.

Sequencing. To further characterize isolates, DNA obtained as described above was taken as a template for another PCR. The annealing temperature started at 60°C; thus, the touchdown protocol consisted of only 10 cycles followed by 19 cycles in which the annealing temperature was 55°C. No GC clamp was added in this step. The PCR product was purified with a GFX PCR DNA and gel band purification kit (Amersham Pharmacia Biotech) following the instruction manuals

of the manufacturer. Sequencing reactions were performed at GATC Biotech AG (Konstanz, Germany). The resulting sequences were analyzed with the online database BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Extraction of Plant Material. Freeze dried plant powder was extracted with 70% aqueous acetone (v/v; 10 ml/pg DW) for 2 hr at 15°C. Suspensions were continuously stirred in an Eppendorf thermomixer (interval mode, 1 min at 1100 rpm, 1 min pause). After centrifugation (10 min, 13,600*g*, 4°C), the supernatant was stored at 4°C, and the pellet was resuspended in acetone and extracted as above. Supernatants were combined and dried in a vacuum evaporator. The residue was redissolved in 50% methanol to a final concentration of 100 μg (DW)/ml.

Bacterial Assays. Bacterial assays were performed to analyze the inhibitory effects of crude plant extracts. We used CPS agar, adjusted with NaOH to pH 8.4 before autoclaving. Crude plant extracts were applied in 5-μl aliquots to agar plates and allowed to dry. Stepwise application of droplets yielded final extract concentrations equivalent to 0.5, 1, and 2 mg of extracted plant material. Controls used 20 μl of 50% methanol. Each extract and each concentration was tested in duplicate. As indicator organisms, isolated bacterial strains from agar plates were suspended in water and their optical density (OD_{600}) was adjusted to 0.1. These suspensions were diluted 1:50 with water in order to get distinct colonies on the plates. Then 0.1 ml of these suspensions was spread on the agar plates and incubated at 24°C for 48 hr. The presence of inhibition zones was recorded and their diameters measured. Bacterial assays were performed with upper leaf extracts from *M. spicatum* and *P. perfoliatus*. We also performed one series with extracts of apical shoot sections of *M. spicatum*, and one series with upper leaves of *M. spicatum*, both using CPS agar with a pH of 7.0 and small Petri dishes (32 mm diam.). In this case, only the highest extract concentration equivalent to 2 mg DW of plant material was used.

A third experiment was performed to verify that polyphenolic compounds in *M. spicatum* were responsible for antimicrobial effects in crude extracts. We first treated extract with polyvinyl polypyrrolidone (PVPP) to remove phenolic compounds. Secondly, we compared the inhibitory strength of extract with pure phenolic compounds applied in equivalent concentrations. PVPP-treatment of the crude extract to remove phenolic compounds was performed as described recently (Gross et al., 1996). The content of phenolic compounds was measured with the Folin-Ciocalteau assay and expressed as tannic acid equivalents; the concentration of tellimagrandin II was quantified by HPLC (Gross et al., 1996). Extract used in experiment III contained 160 μg tannic acid equi valents and 27 μg tellimagrandin II per milligram of plant dry weight. Accordingly, we used 27 and 160 μg of (+)-catechin, gallic acid, and tannic acid (Sigma Aldrich) in the assays. Purified tellimagrandin II was available in only a limited amount, and we used 10 μg per assay. All tests were performed in triplicate.

RESULTS

Bacterial Counts. Bacteria in the gut of *Acentria* may, at least in part, be derived from epiphytic bacteria of their host plants. Therefore, we measured bacterial density on both *M. spicatum* and *P. perfoliatus*. The former had $1.43 \pm 0.83 \times 10^6$ cells/cm^2 and the latter $2.51 \pm 0.89 \times 10^6$ cells/cm^2 surface area ($N = 5$ each; mean $\pm$ SD; Student's t test; $t = 2.17$, $P = 0.055$; Table 1).

Instar stages of *Acentria* larvae can be derived from the size of their head capsule (Gross et al., 2002). Head capsule width ranged from 462 to 897 μm (641 ± 212 μm, $N = 5$) in *P. perfoliatus* and from 550 to 1482 μm (871 ± 337 μm, $N = 6$) in *M. spicatum* fed larvae. Table 1 summarizes densities of bacteria in the fore-, mid-, and hindgut of larvae fed with *P. perfoliatus* or *M. spicatum*. Bacterial counts varied from 0.39 to 10.01×10^6 ($1.9 \pm 1.8 \times 10^6$) cells per gut segment. There was no significant difference between cell numbers in the different gut segments nor was there a difference between cell numbers in larvae raised on different food sources. Bacterial densities did not correlate with head capsule width.

Characterization of Bacterial Isolates. We selected bacterial strains from agar plates based on colony size, morphology, and color. Dominant morphotypes of epiphytic bacteria from both plants were selected for further characterization. This yielded 17 strains. Furthermore, 29 isolates were obtained from guts of larvae fed with *M. spicatum* and 18 from those fed with *P. perfoliatus*. Of the 64 isolates selected based on morphological differences, only 17 were genetically different according to DGGE. These strains were further characterized by sequence analyses (Table 2).

Small subunit rRNA gene sequences of these 17 isolates were compared with the online database BLAST. Sequence similarities ranged between 97 and 100%.

TABLE 1. BACTERIAL COUNTS IN *Acentria* GUTS AND ON MACROPHYTE LEAVES

Treatment	Bacterial counts ($\times 10^6$)[a]	N
Acentria fed with *P. perfoliatus*		
Foregut	1.8 ± 1.2	5
Midgut	2.1 ± 0.9	5
Hindgut	1.3 ± 1.4	4
Acentria fed with *M. spicatum*		
Foregut	3.6 ± 3.5	6
Midgut	1.1 ± 0.8	6
Hindgut	1.7 ± 1.0	6
P. perfoliatus leaves	2.5 ± 0.9	6
M. spicatum leaves	1.4 ± 0.8	6

[a] Values are the mean $\pm$ SD. Counts for gut segments are cells/segment; for leaves, cells/cm^2 leaf area.

TABLE 2. INHIBITION OF BACTERIAL ISOLATES FROM MACROPHYTES AND GUTS BY *M. spicatum* EXTRACTS

Subgroup	Bacterial isolates[a] closest relative	16S rDNA fit (%)	Source[b]				Inhibition zone diameter (mm)[c]				
							pH 7.0[d]		pH 8.4[d] Leaves		
			PFL	MSL	PFG	MSG	Shoot,[e] 2 mg	Leaves, 2 mg	2 mg	1 mg	0.5 mg
α	*Brevundimonas*	100				x	ND	ND	ND	ND	ND
α	*Afipia*	100			x	x	>32	>32	20	12	0
α	*Sphingomonas*	99				x	ND	ND	15	8	0
β	*Comamonas*	97			x	x	20	16	24	20	18
γ	*Aeromonas*	100	x	x	x	x	14	10	6	0	0
γ	*Enterobacter*	100			x		0	0	0	0	0
γ	*Acinetobacter* (sp. 1)	100				x	18	15	ND	ND	ND
γ	*Acinetobacter* (sp. 2)	100				x	18	15	23	16	7,5
γ	*Pseudomonas*	99			x		ND	ND	20	14	10
Gram+ low GC	*Bacillus*	100				x	ND	ND	20	17	10
Gram+ high GC	*Gordonia* (sp. 1)	99			x		22	17	25	22	18
Gram+ high GC	*Gordonia* (sp. 2)	100			x		24	18	31	25	20
Gram+ high GC	*Leucobacter*	97			x		>32	>32	20	15	10
Gram+ high GC	*Rhodococcus*	100			x		ND	ND	ND	ND	ND
Gram+ high GC	*Tsukamurella*	100			x		22	19	25	20	17
CF cluster	previously uncultured	97		x	x		23	19	38	30	24
CF cluster	*Chryseobacterium*	97	x	x		x	20	16	>40	>40	40

[a] As revealed by BLAST.
[b] Source of the respective strains: PFL = *P. perfoliatus* leavs; MSL = *M. spicatum* leavs; PFG = gut of *P. perfoliatus* fed larva; MSG = gut of *M. spicatum* fed larvae.
[c] 1 SD of replicates was always below 1.
[d] pH of agar plates.
[e] Apical shoot sections.

Ten strains showed 100% identity with known bacteria. The four isolates with only 97% homology probably represent previously unknown bacteria. Members of α-, β-, or γ-proteobacteria, the CF cluster (*Cytophaga/Flavobacteria*), and Gram-positive bacteria were identified.

Sequence analyses revealed that only three bacterial strains dominated the cultivable fraction of epiphytic bacterial population. Two of them belonged to the CF cluster. One showed the closest relation to an as yet uncultivated epilithic bacterium, and the other to *Chryseobacterium defluvium*. Both cases exhibited only 97% conformity with described species. The third strain showed 100% similarity with the common freshwater bacterium *Aeromonas veronii*. All three epiphytic strains were also detected in guts of *Acentria*. One of them and two other proteobacteria were found in *Acentria* guts of both treatments (Table 2). A surprisingly high proportion of Gram-positive strains were found in larvae fed with *P. perfoliatus* (5 of 11) compared with larvae fed with *M. spicatum* (1 of 9).

Bacterial Assays. The antimicrobial activity of plant extracts was measured with a modified agar disc diffusion assay. None of the bacterial isolates was inhibited by extracts of *P. perfoliatus*, even when applied in the highest concentration equivalent to 2 mg plant biomass (DW) extracted. Tannin-containing extracts of *M. spicatum* inhibited most strains, even at the lowest concentration used (Table 2). The size of the inhibition zone increased with higher extract concentrations used. Extracts made with apical shoot sections of *M. spicatum* caused larger inhibition zones than those made with upper leaves using the same concentration. HPLC analysis showed that in our extracts apical shoot sections contained 30% more tellimagrandin II based on dry weight compared to upper leaf extracts. Tellimagrandin II is the major hydrolyzable tannin in *M. spicatum* (Gross et al., 1996). The inhibition of bacterial isolates also differed with the pH of the agar plates used and the *M. spicatum* parts extracted. Using extract equivalent to 2 mg plant dry weight, we observed smaller inhibition zones of bacteria on neutral agar plates (pH 7.0) compared to alkaline conditions (pH 8.4) with 9 of 11 strains.

The susceptibility of bacterial isolates towards *M. spicatum* extract varied on alkaline media. Isolates belonging to the α- or γ-proteobacteria exhibited only a weak inhibition. Two of the α-proteobacteria were not inhibited with the lowest extract concentration. One strain, *Enterobacter* sp. (γ-proteobacteria) was not affected at all. Another member of this subgroup, *Aeromonas* sp., was only slightly inhibited with the highest concentration used. Two other γ-proteobacteria (*Acinetobacter* and *Pseudomonas*) exhibited inhibition zones almost as large as those observed for Gram-positive bacteria. The inhibition zone of Gram-positives at the lowest concentration ranged from 10 to 20 mm. Inhibition of the only β-proteobacterium isolated was comparable to that of Gram-positive bacteria. Isolates belonging to the CF cluster exhibited the highest susceptibility towards *M. spicatum* extract.

TABLE 3. INHIBITION[a] OF SELECTED BACTERIAL STRAINS BY *M. spicatum* EXTRACTS AND PHENOLIC COMPOUNDS

	Comamonas	*Pseudomonas*	*Chryseobacterium*
M. spicatum extract (1 mg)	17 ± 0	23 ± 1	23 ± 2
PVPP treated (1 mg)	0	0	14 ± 1
Tannic acid			
160 μg	20 ± 1	22 ± 0	30 ± 1
27 μg	13 ± 2	14 ± 1	19 ± 2
Gallic acid			
160 μg	>40.0	>40.0	>40.0
27 μg	13 ± 0	26 ± 1	23 ± 1
(+)-Catechin (160 μg)	0	0	0
Tellimagrandin II (10 μg)	4 ± 0	9 ± 2	11 ± 0

[a] Mean $\pm$ SD (mm).

The susceptibility of three selected strains were tested with *M. spicatum* extract, extract treated with PVPP, and pure phenolic compounds on alkaline media (Table 3). PVPP treatment caused a disappearance of inhibition in two of three strains and greatly reduced the inhibition in the CF strain. Gallic and tannic acid as well as tellimagrandin II inhibited all selected strains, whereas catechin was inactive. This indicates a high susceptibility of bacterial isolates towards tannins in *M. spicatum.*

DISCUSSION

Our study investigated the allelochemical interactions between freshwater macrophytes, the herbivorous aquatic moth *Acentria ephemerella* and their gut symbionts. We fed larvae of *Acentria* with a tannin-free (*Potamogeton perfoliatus*) or a tannin-rich (*Myriophyllum spicatum*) macrophyte. Tannins are well-known herbivore deterrents (Bernays, 1981), but they also have antimicrobial effects (Scalbert, 1991; Field and Lettinga, 1992). Our hypothesis is that food-derived tannins influence the naturally occurring gut microbiota of lepidopteran larvae, which may lead to an altered performance of the larvae. Larvae exhibited reduced growth when fed with *M. spicatum* (Choi et al., 2002). Results of this study show that gut bacteria of *Acentria* are affected by secondary metabolites of the freshwater macrophytes on which larvae were feeding.

We found evidence for the presence of gut symbionts in larvae of *Acentria*. Direct DAPI counts of bacteria revealed densities of $2.8–13.3 \times 10^6$ cells/gut. Converting this to gut volume (see Methods and Materials section), we determined bacterial densities of $0.3–2.2 \times 10^6$ cells/μl. For other arthropods, $0.07–190 \times 10^6$ cells/μl gut fluid have been reported (Cazemier et al., 1997). To our knowledge,

our study is the first to present quantitative data on bacterial densities in guts of Lepidoptera.

Bacterial numbers did not vary between fore-, mid-, or hindgut, and no correlation between the bacterial counts and the size of the larvae was found. So far, it has not been possible to evaluate which factors influence the density of gut bacteria in Lepidoptera, since so little is known in this field. The large variability observed in bacterial densities of different gut segments or larvae may have several explanations. First, bacterial densities in Lepidoptera may naturally show large fluctuations. A few days before metamorphosis, lysozyme is secreted in the midgut in some Lepidoptera larvae (Russell and Dunn, 1991), which would account for lower densities at that time. Second, the rearing procedure can influence bacterial densities. We observed higher densities of bacteria in larvae kept for a prolonged time in the laboratory compared to larvae taken fresh from the field (data not shown). This indicates that laboratory conditions can alter densities of gut bacteria.

We assume that the gut microbiota in *Acentria* is mainly derived from inoculates through the respective food plants. The bacterial densities on the two plants used in our study differed only marginally (Table 1). We did not observe any difference between the mean abundance of bacteria in larvae fed with *P. perfoliatus* or *M. spicatum*. At least two possibilities could account for this: either, tannins from *M. spicatum* may not affect gut bacteria or, the gut bacterial community may have been shifted to more tannin-tolerant strains.

To investigate the inhibitory effect of plant extracts on *Acentria* gut bacteria, we isolated several strains from larvae on different food treatments and performed agar diffusion assays. Sequence analysis revealed only a little overlap in the bacterial isolates from larvae fed with either *P. perfoliatus* or *M. spicatum* (Table 2). None of the strains were inhibited by extracts of leaves from *P. perfoliatus*, even when high concentrations were used. In contrast, extracts of upper leaves from *M. spicatum* inhibited the growth of all but one strain (Table 2).

The antimicrobial effect of *M. spicatum* extracts is, for the most part, due to polyphenols, as was shown by the PVPP assay and comparison with pure phenolic compounds. At present, we cannot fully exclude that some nonphenolic compounds have additional effects, since one strain (*Chryseobacterium* sp.) still exhibited an inhibition after PVPP treatment of the extract.

These experiments were performed using CPS agar with alkaline conditions (pH 8.4 before autoclaving) to mimic Lepidoptera midgut conditions (Appel and Martin, 1990; Clark, 1999). Alkaline conditions promote the autoxidation of tannins and phenolic compounds of lower molecular weight (Appel, 1993). This enhances their toxicity towards microorganisms (Field and Lettinga, 1992). Accordingly, the inhibition zones were always smaller with all but two strains tested when using neutral CPS agar (pH 7.0, Table 2). The inhibition zones in the assay increased when the same amount of extract from apical shoot sections of

M. spicatum were used. Apical shoot sections contain much higher concentrations of polyphenols, among them tellimagrandin II, as was shown with our extracts and in previous studies (Gross, 2000). This indicates a dose-dependent growth inhibition of bacteria by milfoil polyphenols.

We observed considerable differences in the susceptibility of the strains towards these extracts. In our study, both α- and γ-proteobacteria displayed the weakest inhibition of all isolates. The two strains belonging to the CF cluster (*Cytophaga/Flavobacteria*) exhibited the largest inhibition. We found these strains in high densities on the plants, indicating that they are transient, and that the larvae obtained them with their food. To our knowledge, inhibition of flavobacteria by tannins has not been investigated before.

The high susceptibility of Gram-positive bacteria towards polyphenols is in accordance with previous findings (Henis et al., 1964; Puupponen-Pimia et al., 2001). Gram-negative bacteria are thought to be better protected against tannins because of the structure of their outer membrane (Field and Lettinga, 1992). Gram-positive bacteria with a high GC content were found exclusively in guts from larvae fed with *P. perfoliatus*. We assume that the absence of Gram-positive bacteria with high GC content in guts of larvae fed with *M. spicatum* may be a result of the higher sensitivity towards polyphenols in this freshwater macrophyte.

Our results provide the first evidence for the allelochemical effect of *M. spicatum* tannins on gut symbionts in *Acentria*. Due to the small proportion of culturable bacteria, culture studies like ours do not provide sufficient information to observe bacterial community shifts. Consequently, culture-independent studies are needed to verify these findings for the whole bacterial community.

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OLIGOALGINATE RECOGNITION AND OXIDATIVE BURST PLAY A KEY ROLE IN NATURAL AND INDUCED RESISTANCE OF SPOROPHYTES OF LAMINARIALES

FRITHJOF C. KÜPPER,[1,2] DIETER G. MÜLLER,[2] AKIRA F. PETERS,[1,3] BERNARD KLOAREG,[1] and PHILIPPE POTIN[1,*]

[1]*Station Biologique, UMR 1931*
CNRS-Laboratoires Goëmar
BP 74, F-29682 Roscoff, Brittany, France

[2]*University of Konstanz*
Department of Biology
D-78457 Konstanz, Germany

[3]*Institute for Marine Research*
Düsternbrooker Weg 20
D-24105 Kiel, Germany

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Abstract—Forty-five species of brown algae (Phaeophyceae) were surveyed for their capacity to respond by an oxidative burst to challenges with alginate oligosaccharides. Intertidal frondose brown algae (Fucales) constitutively released high quantities of peroxide. The capacity to recognize oligoguluronates and to react with an oxidative burst was confined to alginate-rich taxa with complex thallus morphology, epitomized by the sporophytes of Laminariales. When kelp sporophytes were impaired in their capacity to perform an oxidative burst by the NAD(P)H oxidase inhibitor diphenylene iodonium, they were readily degraded by their bacterial epiflora. Thus, in these algae, the oxidative response is an essential element of natural resistance. We also report on the establishment of a well-defined experimental system for investigations on kelp immunity, with *Laminaria digitata* as the host and its phaeophycean endophyte, *Laminariocolax tomentosoides*, as the pathogen. We found that an alginate-triggered oxidative burst significantly induces resistance in *Laminaria digitata* against infection. From these findings we infer that oligoalginate signals are important cues in the interaction between laminarialean kelps and potential pathogens.

Key Words—Marine algae, kelps, *Laminaria*, *Laminariocolax*, *Macrocystis*, oligoalginates, oxidative burst, natural resistance, induced resistance.

* To whom correspondence should be addressed. E-mail: potin@sb-roscoff.fr

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INTRODUCTION

In marine macroalgae, competition and predation have been thought of as the major factors determining the structure of natural populations and communities (e.g., Lubchenco and Gaines, 1981; Carpenter, 1990; Norton et al., 1990). Interactions with pathogens were considered too rare to have any significant impact on seaweed populations. Therefore, the importance of defense reactions against pathogens for understanding the functioning of marine ecosystems has been somewhat overlooked, and deciphering host-pathogen interactions in algae is still in its pioneering phase (Correa, 1997; Potin et al., 1999).

Only a few marine pathosystems have been studied in some detail. These are mainly in the Rhodophyta. Weinberger et al. (1999) described an oxidative burst triggered by oligoagars in *Gracilaria conferta*, showing that accumulation of hydrogen peroxide induces the elimination of agar-degrading epiphytic bacteria from the thallus surface (Weinberger and Friedlander, 2000). Bouarab et al. (1999) demonstrated the role of carrageenan sulfation patterns in regulating the virulence of *Acrochaete operculata*, a pathogen of *Chondrus crispus*. *C. crispus* gametophytes reacted with a significant release of hydrogen peroxide in their culture medium when challenged by *A. operculata* cell-free extracts.

With respect to brown algae, we showed recently that oligomeric degradation products of alginate, the main matricial cell wall polysaccharide in this lineage, elicit an oxidative burst in the sporophytes of the kelp *Laminaria digitata* and trigger a cascade of signaling events (Küpper et al., 2001). Alginate is a linear polysaccharide made of homopolymeric blocks of β-1,4-D-mannuronic acid (oligomannuronates) or α-1,4-L-guluronic acid (oligoguluronates), and blocks with mixtures of both monomers (Haug et al., 1974). At micromolar concentrations or less, the homo-oligomeric fragments composed of guluronate residues elicited a transient, yet strong oxidative response, whereas polymannuronate blocks were inactive in *L. digitata* (Küpper et al., 2001).

Laminariales (kelps) are colonized by various morphologically distinguishable bacteria (Pellegrini and Pellegrini, 1982; Corre et al., 1989), including alginate-degrading strains such as *Pseudomonas alginovora* (Boyen et al., 1990a). In Japanese mariculture systems of *Laminaria japonica*, the alginolytic bacterium *Pseudoalteromonas elyakovii* was identified as the causative agent of the wounded-spot disease (Sawabe et al., 2000). In natural populations, kelp sporophytes are also frequently infested by phaeophycean endophytes, resulting in disease symptoms such as warts, crippled thalli, and twisted stipes and blades (Apt, 1988; Kawai and Tokuyama, 1995; Peters and Schaffelke, 1996; Peters and Ellertsdóttir, 1996; Ellertsdóttir and Peters, 1997; Burkhardt and Peters, 1998). Field observations (A. F. Peters, unpublished) suggest that mortality of such endophyte-infested adults is increased, mainly because stipes of infected individuals cannot resist the increased drag. In mature sporophytes, the presence of endophytes inhibits

sorus formation (Peters and Schaffelke, 1996; Lüning et al., 2000). Altogether, pathogenic microorganisms are likely to be an important parameter in the fitness of these brown algae.

Based on the biochemistry of host-pathogen interactions in terrestrial plants and on the above-mentioned observations in the Rhodophyta, one might expect that recognition of alginate oligosaccharides followed by an oxidative response is a key point in the defense strategies of brown algae against microbial epiphytes or pathogens. However, it is not known whether brown macroalgae other than the morphologically relatively complex and perennial *Laminaria digitata* also recognize this elicitor. Hence, we have surveyed a number of brown algal groups, including species and life-history stages with a simple morphology, for their ability to sense the presence of oligoguluronate fragments, as shown by the release of reactive oxygen species (ROS) into their surrounding medium. The potential role of the oxidative burst in the natural resistance of kelps was then further investigated by loss-of-function experiments, using an irreversible inhibitor of the mammalian oxidative burst-generating NAD(P)H oxidase, diphenylene iodonium. Finally, we report on controlled inoculations using a convenient host-pathogen system to assess the biochemical basis of immunity in brown algae, using sporophytes of *Macrocystis pyrifera* and *L. digitata* and their natural endophytic pathogens of the genus *Laminariocolax*.

METHODS AND MATERIALS

Algal and Microbial Materials. We used field specimens of various brown algal species collected from the seashore near Roscoff (Brittany, France) and Kiel (Baltic Sea, Germany). Additional species were grown as unialgal laboratory cultures originating from various localities (Table 1). Axenic sporophytes of *Macrocystis pyrifera* were obtained from a pair of axenic gametophytes from Chile (Magellan Strait). The brown algal endophytes, *Laminariocolax tomentosoides* (Farlow) Kylin (LtoH) and *L. macrocystis* (Peters) Peters (Lmac LM2) have been described previously (Burkhardt and Peters, 1998; Peters, 1991). Algae were grown in Provasoli ES medium (Starr and Zeikus, 1987) prepared with natural seawater. Cultures, illuminated with daylight-type fluorescent lamps at an irradiance of 9 μmol photons/m^2/sec for 10 hr/day, were maintained at 10 $\pm$ 1°C in 10-ml Petri dishes and transferred to fresh medium at 1- or 2-week intervals.

The oomycete *Eurychasma dicksonii* (Wright) Magnus was available in a unialgal culture in its host *Pylaiella littoralis* (L.) Kjellm., as described previously (Müller et al., 1999). The epiphytic bacterium *Pseudomonas alginovora*, strain XO 17 isolated from *Laminaria digitata* (Corre et al., 1989) was grown in ZoBell medium (ZoBell and Upham, 1944) as previously described (Boyen et al., 1990a).

Measurements of Hydrogen Peroxide Levels. Experiments were carried out in a 12°C temperature-controlled room, except for warm-water species (*Dictyota*

TABLE 1. RATES OF RELEASE BY BROWN ALGAL SPECIES OF HYDROGEN PEROXIDE IN ABSENCE OR PRESENCE OF OLIGOGULURONATES (150 μg/ml)[a]

Algal species	Life history stage, morphology	Permanent H_2O_2 concentration in cultures (μM): Culture dishes	Permanent H_2O_2 concentration in cultures (μM): Medium of stock collection	Basal H_2O_2 release	Occurrence of the oxidative burst	Origin of plant material (strain code)[b]
Laminariales						
Chorda filum	macrothalli, sp	ND		−	+	Galicia(1)
Chorda filum	microthalli, gam	♂: 0.72 ± 0.12 ♀: 1.4 ± 1.2	ND	−	−	Helgoland(3)
Laminaria digitata	sp	0.048 ± 0.26		−	++	Roscoff (field-collected old sporophytes); Helgoland(2)
	gam	♂: 0.12 ± 0.0008 ♀: 0.07 ± 0.002	♂: 0.019 ± 0.0003 ♀: 0.004 ± 0.001	−	−	Helgoland(2)
Laminaria hyperborea	sp	ND		field-coll.: ++ culture: -	++	Roscoff (field-collected old sporophytes; young sporophytes cultured from spore release of sori of field-collected plants)
Laminaria ochroleuca	sp	0.11 ± 0.24		field-coll.: ++ culture: +/−	field-coll.: +/− culture: +/+	Roscoff (field-collected old sporophytes; young sporophytes cultured from spore release of sori of field-collected plants)
Laminaria ochroleuca	gam	♀: 0.26 ± 0.22 ♂: 0.38 ± 0.25	ND	−	♀:- ♂:+	Roscoff(3)
Laminaria pallida	sp	ND	ND	−	++	Cape Peninsula, South Africa (cell suspension culture produced from a callus)(3)
	gam	ND	ND	−	−	Cape Peninsula, South Africa(3)

Laminaria saccharina	sp	ND		−	++	Roscoff (old sporophytes, field-collected), Hegoland (young sporophytes)
Macrocystis pyrifera	sp	ND		−	++	Fuerte Bulnes, Chile(2)
Lessonia nigrescens	sp	ND		−	++	Chiloe, Chile(2)
Saccorhiza polyschides	sp			++ (field-collected)	+	Roscoff (field-collected)
Fucales						
Ascophyllum nodosum	thallus fragments			++	−	Roscoff (field-collected)
Bifurcaria bifurcata	thallus fragments			++	+	Roscoff (field-collected)
Cystoseira baccata	thallus fragments			++	−	Roscoff (field-collected)
Fucus evanescens	thallus fragments			++	−	Kiel (field-collected)
Fucus serratus	thallus fragments			++	−	Roscoff (field-collected)
Fucus vesiculosus	thallus fragments			++	−	Kiel (field-collected)
Halidrys siliquosa	thallus fragments			++	−	Roscoff (field-collected)
Himanthalia elongata	thallus fragments			++	−	Roscoff (field-collected)
Pelvetia canaliculata	thallus fragments			+	+/−	Roscoff (field-collected)
Sargassum muticum	thallus fragments			++	+/−	Roscoff (field-collected)
Desmarestiales						
Desmarestia aculeata	sp	0.64 ± 0.04		++	+/−	Helgoland(1,2)
Desmarestia dudresnayi	sp	ND		−	+	Galicia(1)
Desmarestia ligulata	sp	ND		++	+	Galicia(1), Roscoff (field-collected)
Desmarestia munda	gam	0.932 ± 0.1	0.09 ± 0.037	++	−	Bamfield, British Columbia, Canada(2)
Scytohamnales						
Splachnidium rugosum	microthalli	4.6 ± 0.01	0.015 ± 0.001	+	−	Tasmania(2)
Dictyotales						
Dictyota dichotoma	sterile, macrothalli			+	−	Roscoff (field-collected)
Dictyota phlyctaenodes	sterile, macrothalli	0.57 ± 0.17	1.28 ± 0.04	+	−	Juan Fernandez Islands(2)
Cutleriales						
Cutleria multifida	gam (♀)	1.33 ± 0.079	0.9 ± 0.025	+	−	Villefranche(2)
Sporochnales						
Sporochnus pedunculatus	gam	0.98 ± 0.04	0.18 ± 0.02	+	−	Villefranche, France, Mediterranean Sea(2)

TABLE 1. CONTINUED

Algal species	Life history stage, morphology	Permanent H_2O_2 concentration in cultures (μM): Culture dishes	Permanent H_2O_2 concentration in cultures (μM): Medium of stock collection	Basal H_2O_2 release	Occurrence of the oxidative burst	Origin of plant material (strain code)[b]
Tilopteridales						
Tilopteris mertensii	nonsexual life history, macrothalli	0.9 ± 0.02	0.14 ± 0.034	+	−	Helgoland(2)
Syringodermatales						
Syringoderma phinneyi	gam	0.35 ± 0.03	0.006 ± 0.0002	+/−	−	British Columbia, Canada(2)
Sphacelariales						
Cladostephus spongiosus	ND/sterile			+	−	Roscoff (field-collected)
Sphacelaria rigidula	sp	0.27 ± 0.016	0.007 ± 0.001	+	−	California(2)
(incertae sedis)						
Microzonia velutina	microthalli	0.69 ± 0.003	0.2 ± 0.01	++	−	Portobello, New Zealand(2)
Zosterocarpus oedogoniae	unknown	0.81 ± 0.014	0.06 ± 0.0001	++	−	Villefranche, France, Mediterranean Sea(2)
Ectocarpales						
Adenocystis utricularis	macrothalli	0.9 ± 0.03	ND	++	−	Ushuaia, Argentina(1)
Chordaria flagelliformis	microthalli	0.074 ± 0.009	0.077 ± 0.024	−	−	Helgoland(1)
Ectocarpus fasciculatus	partheno-sp. (♀), filamentous macrothalli	0.17 ± 0.006	0.14 ± 0.07	+	−	Roscoff(2)
Ectocarpus fasciculatus	sp, filamentous macrothalli	$0.36 \pm 0.01.5$	0.0035 ± 0.0002	+	−	Ireland(2)
Ectocarpus fasciculatus + virus	sp, virus-infected, sterile, filamentous macrothalli	2.39 ± 0.135	0.002 ± 0.0004	+	−	Roscoff(2)

Ectocarpus fasciculatus + virus	sp, sterile, virus-infected, filamentous macrothalli	0.4 ± 0.04	0.0018 ± 0.0001	+	−	Ireland(2)
Ectocarpus siliculosus	gam (♂), filamentous macrothalli	1.25 ± 0.116	0.318 ± 0.056	+	−	Kaikura, New Zealand(2)
Ectocarpus siliculosus + virus	gam, filamentous macrothalli	5.2 ± 0.069	0.61 ± 0.197	+	−	Kaikura, New Zealand(2)
Ectocarpus siliculosus	sp, filamentous macrothalli	0.0049 ± 0.005	0.045 ± 0.004	+	−	Port Aransas, Texas(2)
Elachista stellaris	macrothalli	0.3 ± 0.1	0.13 ± 0.005	−	+/−	Villefranche, France, Mediterranean Sea(2)
Eudesme virescens	microthalli	2.8 ± 0.25	0.18 ± 0.05	+/−	−	Aran Islands, Ireland(2)
Feldmannia irregularis	nonsexual, filamentous macrothalli	1.03 ± 0.14	0.712 ± 0.016	+	−	Canary Islands(2)
Feldmannia irregularis + virus	nonsexual, virus-infected, sterile, filamentous macrothalli	1.46 ± 0.28	0.866 ± 0.017	+	−	Canary Islands(2)
Feldmannia simplex	nonsexual, filamentous macrothalli	1.44 ± 0.0008	0.022 ± 0.0072	+	−	Aran Islands, Ireland(2)
Feldmannia simplex + virus	nonsexual, filamentous macrothalli	0.8 ± 0.008	0.013 ± 0.001	+	−	Aran Islands, Ireland(2)
Myriotrichia clavaeformis	macrothalli	3.98 ± 0.06	0.16 ± 0.074	−	−	Las Grutas, Argentina(2)
Myriotrichia clavaeformis + virus	macrothalli, sterile, virus-infected	1.9 ± 0.02	0.046 ± 0.01	−	−	Las Grutas, Argentina(2)

TABLE 1. CONTINUED

Algal species	Life history stage, morphology	Permanent H_2O_2 concentration in cultures (μM): Culture dishes	Permanent H_2O_2 concentration in cultures (μM): Medium of stock collection	Basal H_2O_2 release	Occurrence of the oxidative burst	Origin of plant material (strain code)[b]
Petalonia fascia	microthalli	1.7 ± 0.2	0.014 ± 0.005	+/−	−	Villefranche, France, Mediterranean Sea(2)
Pylaiella littoralis	nonsexual, filamentous macrothalli	0.11 ± 0.02	0.104 ± 0.06	+/−	+	Isla Diego Ramirez, Chile(2)
Pylaiella littoralis + *Eurychasma dicksonii*	nonsexual, filamentous macrothalli	1.66 ± 0.009		−	+	Shetland (pathogen), Isla Diego Ramirez (host)(2)
Pylaiella littoralis + virus	sterile, virus-infected, filamentous macrothalli	0.53 ± 0.005	0.07 ± 0.003	+	+/−	Isla Diego Ramirez (virus-infected in lab culture)(2)
Scytosiphon lomentaria	microthalli, gam (♀)	0.6 ± 0.08	0.28 ± 0.04	+	−	Victoria, Australia(2)
Spermatochnus paradoxus	microthalli	3.33 ± 0.11	ND	−	+/−	Villefranche, France, Mediterranean Sea(2)

[a] Basal hydrogen peroxide release was determined by following the concentration in dishes of control plants over 3 hr, while permanent concentrations refer to measurements in the culture vessels in which the algae had been kept for at least one week. In some cases, culture medium from a stock culture collection was examined, in which algal samples were untouched for a minimum of one year in 4 ml of culture medium. Where appropriate, significance of results was verified by a *t* test. The relative strength of the oxidative burst after oligoguluronate addition is indicated by: ++, peak H_2O_2 at least 10 times the basal release, $P < 0.01$; +, peak H_2O_2 at least 5 times the basal release ($P < 0.01$, +/−, $0.01 < P < 0.05$), and −, none of the previous logic conditions fulfilled, no significant response. In cases where no oxidative response to alginate oligosaccharides was observed for all the tested species for a given genus, at least one species was also elicited with valinomycin, which had been shown to activate the burst-producing machinery downstream of GG recognition (Küpper et al., 2001). Abbreviations: gam = gametophytes, sp = sporophytes.

[b] Origin of plant material/culture collections: (1) Akira F. Peters, Kiel; (2) Dieter G. Müller, Konstanz; (3) IM = Ingo Maier, Konstanz.

phlyctaenodes, Zosterocarpus oedogoniae), which were tested at 20°C. Hydrogen peroxide concentrations were measured using the luminol chemiluminescence method with a Lumat LB 9507 luminometer as described by Küpper et al. (2001). Both the total amounts of hydrogen peroxide in the medium around plants and the kinetics of H_2O_2 release by brown algal cultures (50–500 mg blotted fresh weight in 10 ml of 0.22-μm-filtered seawater) either in the presence or in the absence of oligoguluronates (150 μg/ml) were monitored, in triplicate, consisting of sampling seawater from three dishes containing the same ratio of biomass/volume of medium. Negative results with oligoguluronates were validated by treatment with the K^+ ionophore valinomycin (5 μM). This potassium carrier had previously been shown to activate the burst-producing machinery downstream of GG recognition (Küpper et al., 2001). Aliquots of 400 μl (out of 10-ml samples) were taken per measurement. All assays were evaluated using two-tailed, paired sample *t* tests.

Cell-free extracts of *Laminariocolax tomentosoides* were prepared as follows. Unialgal cultures were blotted dry on filter paper, weighed, frozen in liquid nitrogen, and ground in an Eppendorf tube with a micropestle and Fontainebleau sand. To a fresh weight of ca. 20 mg algae, 300 μl of extraction buffer (50 mM Tris HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM PMSF; all from Sigma) were added. The samples were vortexed for 1 min, incubated for 5 min (4°C), and centrifuged for 5 min at 10,000*g*. The supernatant was recovered and stored at −20°C until use. Fifty μl of this extract was added to the *L. digitata* sporophytes either 1 hr prior to, or simultaneously with, the addition of alginate oligosaccharides. A similar procedure was used to prepare cell-free extracts from *Pylaiella littoralis* infected or not by *Eurychasma dicksonii.*

Challenge with Epiphytic Bacteria and Endophytes. The involvement of the oxidative burst in the natural resistance of kelps to their natural epiflora was assessed as follows. Axenic sporophytes of *Macrocystis pyrifera*, 2–5 mm in thallus length and in groups of five individuals, were incubated for 10 min in the presence of the NAD(P)H oxidase inhibitor diphenylene iodonium (1, 2.5, 5, and 10 μM from a 1 mM stock solution in DMSO; from Sigma), and thereafter transferred to fresh medium. Some plants were elicited with oligoguluronates at 150 μg/ml for 1 hr before the DPI treatment. The thalli were then inoculated with *Pseudomonas alginovora* (4.5×10^8 colony forming units per 3.1 ml well), and inspected daily for the occurrence of lesions. Alternatively, the natural bacterial epiflora of *M. pyrifera* was reestablished by adding a droplet of culture medium containing *Macrocystis*-associated bacteria from Chile. For comparison, unialgal sporophytes of *L. digitata* (i.e., cultures containing the natural bacterial epiflora from the start) were also subjected to the same DPI treatment.

To address the potential role of oligoalginate signals in the induced resistance of kelps, *L. digitata* sporophytes 2–20 mm in thallus length were elicited with oligoguluronates (GG) (150 μg/ml) for 1 hr. The GG was added to three replicates of groups of five plantlets. After GG treatment, the thalli were transferred to 50 ml

fresh culture medium. The GG treatment was repeated at daily intervals for 14 days, each day to a new set of 15 thalli. Then, all *L. digitata* sporophytes were inoculated with *L. tomentosoides* zoospores, as follows. A culture of sporulating filamentous endophytes in a volume of 500 μl was added to each dish of *Laminaria*. Controls consisted of eight replicates of algae untreated prior to endophyte inoculation and of six replicates of GG-treated sporophytes, which were not inoculated with the pathogen. After coculture for 3 days, the *Laminaria* sporophytes were transferred to fresh medium, which was renewed weekly, and they were examined at 3-day intervals until 3 weeks after the inoculation.

The intensity of disease symptoms and the state of the plants was judged by an unbiased, independent observer, according to the following relative scale: 0, no symptoms of pathogen infection nor unusual necroses; 1, infections and/or necroses only very marginal; 2, few infections or necroses: overall plants in good shape; 3, strong infections or necroses in at least one plant, but other plants of the same batch in good shape; 4, strong infections and necroses in several plants; 5, strong infections, at least one plant dead; and 6, massive infections, all plants dead.

The scores of the replicates of days 1–3, 4–6, 7–9, 10–14 before inoculation of endophytes were grouped and statistically analyzed using a one-way ANOVA with days before addition of endophytes as factor. Multiple mean comparisons were made with the Tukey-Kramer test. After the experiments, all plants were fixed and mounted on paper. They were deposited in the herbarium of the Marine Station, Roscoff.

RESULTS

Oxidative Response in Brown Algae to Treatment with Oligoguluronates. The 45 species belonging to 11 orders of brown algae were monitored for levels of hydrogen peroxide in their surrounding medium, in the absence or presence of alginate oligosaccharides (Table 1). In members of the order Laminariales, steady-state peroxide concentrations in the oligoalginate-free culture dishes were usually below 1 μM for the gametophytes, and below 500 nM for the sporophytes. In *L. digitata* and *L. ochroleuca* sporophytes, the H_2O_2 concentrations were typically in the range of 200 nM. Only in the culture of female *Chorda filum* gametophytes were higher concentrations measured (Table 1).

When challenged with oligoguluronates, culture-raised, young sporophytes of *Chorda filum*, sporophytes of all the *Laminaria* species tested, as well as those of *Macrocystis pyrifera*, *Lessonia nigrescens*, and *Saccorhiza polyschides* reacted with a rapid, strong oxidative burst. *Macrocystis pyrifera* sporophytes (Figure 1A) displayed a maximum of H_2O_2 release 10 times higher than the basal emission ($P \leq 0.01$). Rates of H_2O_2 accumulation were typically as high as 0.3–0.5 μmol/g fresh weight (FW) for centimeter-long parenchymatic thalli within 15 min of the addition of alginate oligosaccharides (due to the higher surface–volume ratio,

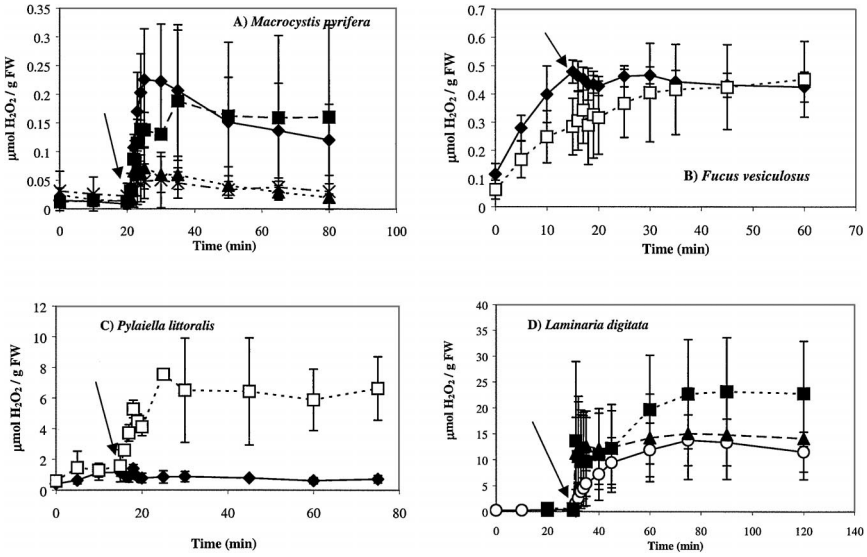


FIG. 1. Hydrogen peroxide concentrations in the culture medium of various brown algae, in the presence or absence of alginate oligosaccharides (GG blocks, 150 μg/ml). Addition of elicitors is marked by an arrow. (A) *Macrocystis pyrifera* sporophytes (Laminariales): GG only, diamonds; 1 μM DPI, squares; 2.5 μM DPI, triangles; 5 μM DPI, asterisks. Note that DPI suppressed the GG-triggered oxidative burst. (B) *Fucus vesiculosus* (Fucales): control, diamonds; GG, squares. (C) *Pylaiella littoralis* (Ectocarpales), control, diamonds; GG, squares. (D) *Laminaria digitata* (Laminariales), challenged with 150 μg/ml GG (circles), cell-free extracts from healthy *Pylaiella littoralis* (triangles) or from *Pylaiella littoralis* infected with *Eurychasma dicksonii* (squares). Error bars indicate standard deviation of the average concentration of H_2O_2 measured in three replicates.

this value could be up to an order of magnitude higher for smaller thalli). In the case of *L. digitata*, *L. ochroleuca*, and *L. pallida*, accumulation of H_2O_2 was very transient, with complete scavenging accomplished around 30 min after the addition of the elicitor. Large (i.e., several meters long) field-collected sporophytes of *L. hyperborea*, *L. ochroleuca*, and *Saccorhiza polyschides* exhibited high constitutive rates of peroxide release and were less or not at all responsive to the elicitor. The minimum size for *Laminaria digitata*, *Lessonia nigrescens*, and *Macrocystis pyrifera* sporophytes to respond with an oxidative burst following incubation with alginate oligosaccharides was 1 mm. Smaller, embryonic sporophytes did not react. With the exception of *L. ochroleuca*, gametophyte cultures of Laminariales did not exhibit an oxidative response following incubation with alginate oligosaccharides.

Outside the Laminariales, the macroscopic thalli of only a few species were found to react by an oxidative burst upon challenge with alginate oligosaccharides.

In the Fucales, all of the species tested constitutively released considerable amounts of hydrogen peroxide. Only *B. bifurcata*, which displayed a maximum of H_2O_2 release more than 5 times higher than the basal emission ($P \leq 0.01$), *P. canaliculata*, *S. muticum* ($0.01 < P \leq 0.05$) and, in few cases, *Fucus vesiculosus* exhibited an enhanced peroxide emission upon addition of oligoguluronates (Table 1, Figure 1B). Among the Desmarestiales, only *Desmarestia dudresnayi* reacted strongly to incubation with the elicitor, while *D. aculeata* and *D. ligulata* showed much weaker oxidative responses (Table 1).

Among the Ectocarpales with erect filamentous thalli, *Pylaiella littoralis* was unique in performing a relatively strong and rapid oxidative burst after the addition of oligoguluronates (Figure 1C); and displayed a peak of H_2O_2 release 5 times higher than the basal emission ($P \leq 0.01$). *Eurychasma*-infected cultures of *P. littoralis* maintained steady-state peroxide levels one order of magnitude higher than healthy cultures. Virus infection in *Pylaiella* coincided with a weaker response to elicitor treatment. The filamentous stages of *Elachista stellaris* and *Spermatochnus paradoxus* displayed weak rates of peroxide release, although *S. paradoxus* maintained fairly high peroxide levels in long-term cultures. They also exhibited a rapid, but comparatively weak, reactivity upon elicitor addition. No other species of Ectocarpales tested showed a burst, with no difference due to the presence or absence of virus infections. After challenging with oligoalginate elicitors, no oxidative response was observed in microthalli of the Scytothamnales, Sporochnales, and Syringodermatales, in exact filamentous thalli of the Tilopteridales and Cutleriales, or in the two filamentous species *Microzonia velutina* and *Zosterocarpus oedogoniae* (Table 1).

Inhibition of Oxidative Burst Abolishes Natural Resistance of Kelp Sporophytes towards Epiphytic Bacteria. To further investigate the potential role of the oxidative response in the control of microorganisms by brown algae, sporophytes of Laminariales were subjected to loss-of-function experiments as follows. The capacity to perform an oxidative burst was suppressed with an NAD(P)H oxidase inhibitor, diphenylene iodonium (DPI), and thalli were then challenged by epiphytic bacteria. As shown in previous experiments with *L. digitata* (Küpper et al., 2001) and in Figure 1A for *M. pyrifera*, DPI completely suppressed the oligoalginate-induced oxidative reaction in kelp sporophytes.

In a first series of experiments (Table 2), axenic sporophytes of *M. pyrifera* were inoculated with the epiphytic alginolytic bacterium, *Pseudomonas alginovora*. Controls with bacteria-free *M. pyrifera* sporophytes confirmed that the treatment with DPI up to concentrations as high as 10 μM had no direct toxic effect on the plants. In the absence of DPI and upon cocultivation with the bacterium, *M. pyrifera* sporophytes also survived for several weeks, without any signs of disease. In contrast, the DPI-treated axenic sporophytes were rapidly attacked by the bacterium, resulting in decoloration within 3–5 days and, finally, complete disintegration at ca. 10 days after the addition of DPI and inoculation (Table 2).

TABLE 2. EFFECT OF NAD(P)H INHIBITOR DIPHENYLENE IODONIUM (DPI) ON INTERACTION BETWEEN *Macrocystis pyrifera* AND EPIPHYTIC BACTERIUM *Pseudomonas alginovora* STRAIN XO 17[a]

Treatment	Bacterial challenge	*N*	Observations
Axenic, untreated control	−	5	normal pigmentation
	+	5	normal pigmentation
DPI, 10 min 1 μM	−	5	plants normal, few apically bleached
	+	5	plants seriously damaged, except a few stipes and meristems completely bleached
2.5 μM	−	5	normal pigmentation and growth
	+	5	plants entirely bleached except a few stipes and meristems
5 μM	−	5	some plants are bleached (except stipes), one half in normal condition
	+	5	all plants bleached except stipes, some are less damaged than those exposed to 10 μM DPI + bacteria
10 μM	−	5	plants in good shape, normal pigmentation
	+	5	strongly bleached, incl. stipes

[a] The observations recorded here are representative for 7 replicated experiments with similar results. The + and − signs refer to the presence or absence of inoculum, respectively. *N*: number of plants tested for a given condition.

Similar experiments were performed with nonaxenic cultures of young sporophytes of *L. digitata*. Even after moderate DPI treatments (5 μM for 1 hr), *L. digitata* plantlets were rapidly (within 3 days) attacked by their natural bacterial epiflora, displaying symptoms similar to the decaying of axenic, DPI-treated *Macrocystis* plantlets artificially inoculated with *Pseudomonas alginovora*. Similar results were also observed when *M. pyrifera* sporophytes were reinoculated with their natural bacterial epiflora (data not shown).

Development of Model Pathosystem for Assessment of Induced Resistance in Kelp Sporophytes. In an attempt to identify a suitable pathogen for artificial infection experiments, kelp sporophytes were challenged with the oomycete *Eurychasma dicksonii*, which had been shown to infect members of many orders of brown algae, including both gametophytes and embryonic sporophytes of *L. digitata* (Müller et al., 1999). However, no artificial infection could be established with *E. dicksonii* in *L. digitata* sporophytes with a size greater than 1 mm. In contrast, two brown algal endophytes of the genus *Laminariocolax* (Ectocarpales), *L. tomentosoides* and *L. macrocystis*, did produce infections in a reliable and reproducible manner. When *L. digitata* or *M. pyrifera* plantlets, 2–20 mm in size, were inoculated with sporulating *Laminariocolax* filaments, the pathogens grew both endo- and epiphytically (Figure 2A,B). *Laminariocolax* caused serious growth perturba-

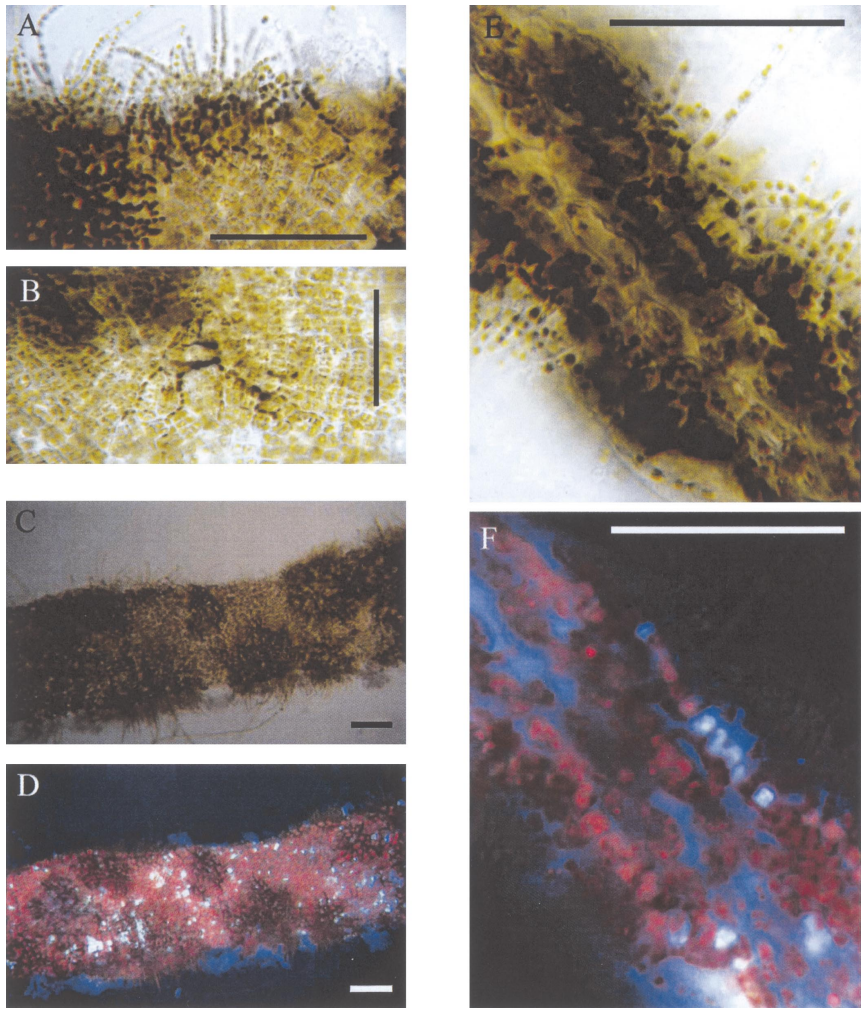


FIG. 2. Infection of *Laminaria digitata* by the endophyte *Laminariocolax tomentosoides* (all scale bars correspond to 100 μm). Superficial views of *Laminariocolax*-infected *L. digitata* plants showing that the pathogen lives both epiphytically (A) and endophytically, as filaments in the intercellular spaces (A, B). (C) On heavily infected plantlets, dense tufts of *Laminariocolax* are visible. (D) Under UV excitation massive necroses are visible as regions with bright blue autofluorescence. (E, F) Section of a strongly infected *L. digitata* plantlet, densely covered with *Laminariocolax* filaments. Penetration of *Laminariocolax* filaments is often accompanied by accumulation of bright blue autofluorescent compounds in the surrounding cortical host cells. *Laminariocolax* filaments in the medulla of this *Laminaria* plant are visible by their darker red plastid fluorescence compared to the brighter red fluorescence of *Laminaria*.

tions of host thalli, finally overgrowing its hosts, with dense tufts of *Laminariocolax* on the heavily infected plantlets (Figure 2C), accompanied by massive necroses (Figure 2D), and frequently resulting in thallus death. Under UV-light observation, the penetration or protrusion zones of infecting *Laminariocolax* filaments (Figure 2E) showed an accumulation of bright-blue autofluorescent compounds (Figure 2F). The first symptoms, consisting of local necroses, were visible within 10 days of inoculation, but the extent of infection was best assessed at one month after inoculation.

Laminariocolax tomentosoides and *L. macrocystis* were found to be cross-infective and attacked both *M. pyrifera* and *L. digitata*. Cell-free extracts of neither brown algal endophyte induced accumulation of H_2O_2 in *L. digitata* sporophyte cultures instead, addition of the cell-free extracts 60 min prior to elicitation with 150 μg/ml oligoguluronates considerably inhibited the oxidative burst (Figure 3A). Cell-free extracts of *Laminariocolax* indeed exhibited a high peroxide-scavenging capacity. Typically, 100 μl extract per 10 ml of seawater degraded 10 μM hydrogen peroxide to over 95% within less than 5 hr, 50% within the first 15 min. However, reactivity of *Laminaria* sporophytes was reestablished after transfer to fresh medium lacking endophyte extract (Figure 3B). In contrast, cell-free-extracts from the nonpathogenic ectocarpoid *Pylaiella littoralis* triggered an oxidative burst response in *L. digitata*, while extracts of *Pylaiella littoralis* were twice as active when the alga was infected by the oomycete *Eurychasma dicksonii* (Figure 1D).

Alginate Oligosaccharides Induce Resistance in Laminaria digitata against *Laminariocolax tomentosoides*. The potential role of the oxidative burst in protecting *Laminaria* sporophytes from infections by brown algal endophytes was next addressed, by treating *L. digitata* plantlets with alginate oligosaccharides at a concentration of 150 μg/ml, followed by challenge with *L. tomentosoides*. Nonelicited sporophytes inoculated with the endophyte displayed the symptoms described above (Figure 2 and 4A–D), i.e., dense infestation by endo- and epiphytic *Laminariocolax* filaments and conspicuous necrotic lesions. In contrast, young sporophytes that had been incubated for 2 hr in the presence of oligoguluronates, allowed to grow for 8 days in normal seawater medium, and then challenged by *L. tomentosoides* sporulating filaments were remarkably well protected against infection. Very few if any epi- or endophytic filaments were visible and even fewer necroses occurred (Figure 4E–H). Remarkably, while untreated plants showed a strong, localized accumulation of blue autofluorescent compounds in the cortical cells around penetrating *Laminariocolax* filaments (Figure 4D), the oligoguluronate-treated plants exhibited a less intense, but ubiquitous cortical accumulation of blue-autofluorescent compounds (Figure 4H).

Using a relative scale to score the extent of infection and various time intervals between elicitation of *Laminaria digitata* thalli with alginate oligosaccharides and inoculation by *Laminariocolax tomentosoides*, we next investigated the time required for the induction of resistance and its duration.

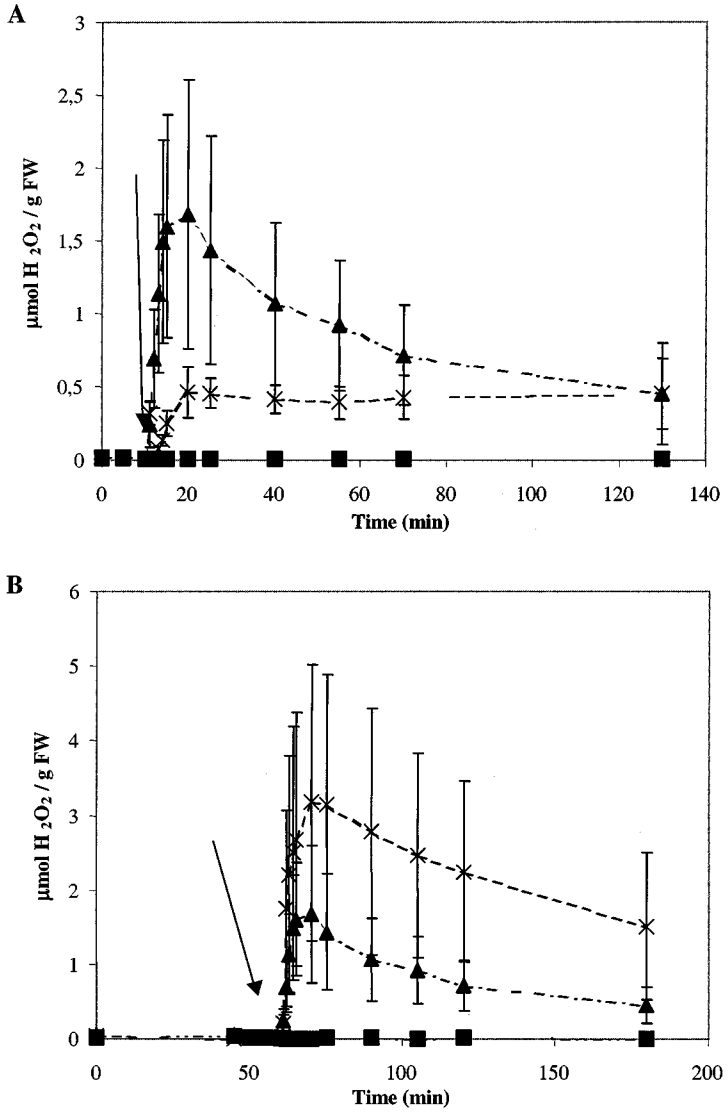


FIG. 3. *Laminariocolax tomentosoides* cell-free extracts scavenge the reactive oxygen species generated by the oxidative burst in *Laminaria digitata* (addition of 150 μg/ml GG is marked by an arrow). (A) *Laminariocolax* extracts added 1 min after GG elicitation, asterisks; control, squares; GG only, triangles. (B) Cell-free extracts were added for 45 min, medium was changed completely, and the elicitors were added 15 min later. In contrast to the above, the treated plants (asterisks) were unhindered in their oxidative burst response. Error bars indicate standard deviation of the average concentration of H_2O_2 measured in 3 replicates.

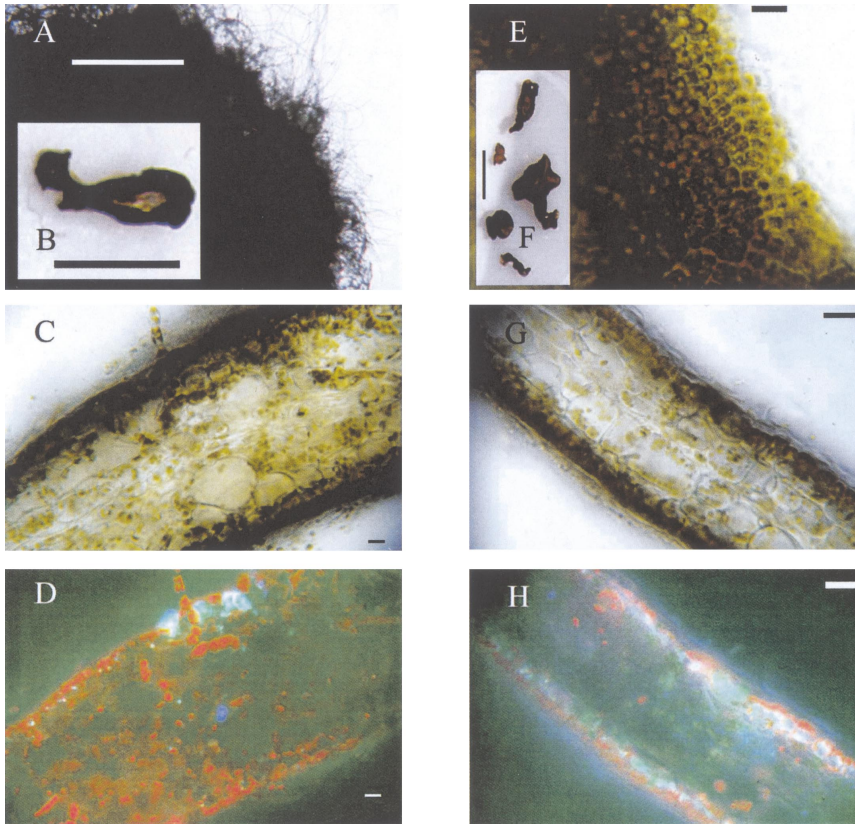


FIG. 4. Oligoguluronates induce resistance to *L. tomentosoides* in *L. digitata*. In controls (A–D), plantlets are completely overgrown (A, B), and frequently penetrated by (C, D) *Laminariocolax* filaments. As shown also in Figure 2, penetrating filaments are often surrounded by bright blue autofluorescent compounds (D). *Laminaria digitata* plantlets were elicited with alginate oligosaccharides for 2 hr with 150 μg/mlGG, grown for 9 days in PES medium, then inoculated with *Laminariocolax tomentosoides* (E–H). Hardly any epi- or endophytic *Laminariocolax* filaments were visible, and the cortex of GG-treated plants often showed accumulation of autofluorescent compounds, although this was less intense and much more widespread and diffuse than in untreated plants. Photographs were taken 34 days after inoculation with the pathogen. Scale bars: A, 1 mm; B, F: 1 cm; C, D, E, G, H: 10 μm.

There was a statistically significant difference in the intensity of symptoms between plantlets subjected to the different treatments (ANOVA, $F = 9.254$, $P = 0.0001$). Treatments with oligoguluronates more than 7 days before endophyte inoculation were significantly less infected than controls untreated before

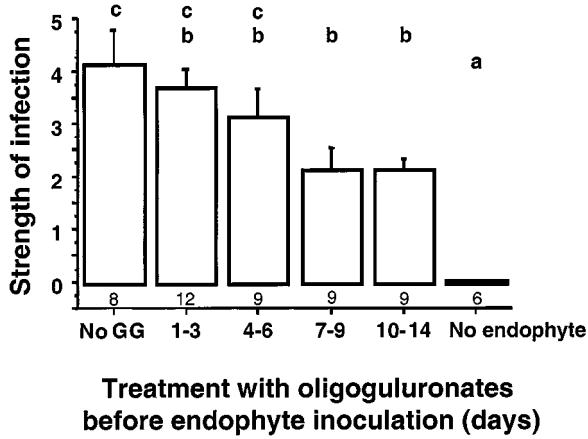


FIG. 5. Strength of the infection of *Laminaria digitata* by the endophyte *Laminariocolax tomentosoides* after treatment with oligoguluronates at various intervals before pathogen inoculation. At 3 weeks after inoculation, relative intensity of symptoms, from 0 (complete resistance) to 6 (highest strength of infection, see Methods and Materials section), was determined by an independent observer. Error bars indicate variability of strength of infection, expressed as the standard deviation of the marks attributed to a given series of plants. Analysis was by one-way ANOVA (see Methods and Materials). Letters above the bars denote significant groupings based on Tukey-Kramer multiple-comparison tests ($P < 0.05$). N below the bars indicates number of replicates.

challenge with the pathogen (Figure 5). Interestingly, the reduction of the strength of infection was progressive and the treatments with oligoguluronates less than 7 days before inoculation showed weaker resistance. Although the treatment with oligoguluronates did not prevent the occurrence of some minor symptoms, it reduced their intensity and conferred a protection that persisted for >7 days.

DISCUSSION

In any host–pathogen interaction, recognition of the occurrence of an attack is the first line of defense. This may involve recognition of the attacker, through either the perception of exogenous signals (defined as exogenous elicitors of defense responses), or of degradation products from the host itself (endogenous elicitors), or both. Therefore, in Laminariales, the oxidative response to the perception of alginate oligosaccharides is probably related to the sensing of damage by microorganisms of the alginate-rich cell wall matrix of kelps. Among the natural bacterial epiflora of Laminariales, many strains have been

reported to harbor alginate-degrading enzymes. These include nonpathogenic bacteria, such as the one used in this study, *Pseudomonas alginovora* (Boyen et al., 1990a), and pathogenic strains, such as the agent of the spot-wounded disease in *L. japonica*, *Pseudomonas elyakovii* (Sawabe et al., 2000). No alginolytic activities have so far been reported in brown algal endophytes. However, scanning electron microscopy investigations of the early stages of the development of *Laminariocolax* and *Laminarionema* zoospores suggest that their mode of penetration may involve the enzymatic degradation of host surfaces rather than a mechanical disruption (Heesch and Peters, 1999). Finally, alginases have been detected in marine fungi (Wainwright and Sherbrock-Cox, 1981) as well as in a variety of kelp grazers, including mollusks and echinoderms (see Boyen et al., 1990b). On the other hand, oligoguluronate signaling may also be related to the need for recognizing challenge by alginate-containing brown algal endophytes and epiphytes.

Elicitor recognition, at the surface or in the cytosol of host plant cells (Ebel and Mithöfer, 1998), is followed within minutes by a so-called oxidative burst, which consists of the accumulation of ROS, such as superoxide ions, hydrogen peroxide, or hydroxyl radicals, via the activation of plasma membrane-associated NAD(P)H oxidases or apoplastic peroxidases. Several, convergent lines of evidence emerging from this study show that accumulation of ROS is an essential component of the defense system of kelp sporophytes against potentially harmful microorganisms. In loss-of-function experiments, in which the capacity of performing an oxidative burst was suppressed by the NAD(P)H oxidase inhibitor diphenylene iodonium, axenic sporophytes of *Macrocystis pyrifera* became very susceptible to the alginate-degrading, yet naturally nonpathogenic bacterium, *Pseudomonas alginovora*. Similarly, control of the bacterial epiflora by nonaxenic sporophytes of *Laminaria digitata* was shown to depend on a DPI-sensitive mechanism, because this kelp species is similarly capable of triggering an oxidative response following recognition of alginate oligosaccharides (Küpper et al., 2001). It follows that, in Laminariales, the capacity of releasing activated oxygen species appears as a central, causal element for keeping the bacterial epiflora at a tolerable level. This conclusion is consistent with previous findings showing that apoplastic peroxide concentrations, such as those occurring during an oxidative burst, can inhibit the growth of kelp-associated bacteria (Küpper et al., 2001). It suggests that activated oxygen species may have a direct role in controlling the growth of potentially pathogenic microorganisms. Such a biochemical basis for the interaction between kelps and alginolytic bacteria is reminiscent of the ecological relationship between red algal agarophytes and their agar-degrading epiphytic bacteria, which also depends on the capacity of the host to perform an oxidative response following recognition of agar oligosaccharides (Weinberger and Friedlander, 2000; Weinberger et al., 2001).

Reciprocally, in gain-of-function experiments by incubation with alginate oligosaccharides, elicited *L. digitata* sporophytes became resistant to infection by

their natural endophytic pathogen *Laminariocolax tomentosoides*. A 1-hr incubation in the presence of alginate oligosaccharides is known to induce a strong emission of H_2O_2 in *L. digitata* (Küpper et al., 2001), further indicating that the oligoguluronate-induced oxidative burst also plays a central role in the induction of resistance to the endophyte. This observation in kelp sporophytes is in agreement with the report that the oxidative burst in *Chondrus crispus* is a key element in establishing resistance to its green algal pathogenic endophyte *Acrochaete operculata* (Bouarab et al., 1999) and also with studies indicating a major role of H_2O_2 in the induction of systemic acquired resistance in higher plants (Alvarez et al., 1998; Van Camp et al., 1998).

Other observations in this study indirectly support the above conclusions. In natural populations, the sporophytes of Laminariales can show a nearly 100% prevalence of pathogenic endophytes, with severe disease symptoms such as warts, crippled thalli, and twisted stipes (Ellertsdóttir and Peters, 1997). We show here (Figure 3) that, unlike extracts of the closely related ectocarpoid, *Pylaiella littoralis*, *Laminariocolax* cell-free extracts not only are not recognized by *L. digitata* sporophytes, but also are capable of quenching the alginate-induced oxidative response. This finding suggests that the infective success of these endophytic pathogens may be due to their capability to avoid recognition or even to suppress the defenses of the host. In contrast to *Laminariocolax*, *Eurychasma dicksonii* is fended off by the sporophytes of Laminariales, whereas it is a natural pathogen to a large variety of brown algal groups (Küpper and Müller, 1999; Müller et al., 1999). As suggested in Figure 1D, this oomycete may produce compounds that are specifically recognized by *L. digitata*. Remarkably, also, the virulence of this pathogen correlates well with the inability of its brown algal hosts to perform an oxidative burst. In particular, the threshold size (ca. 1 mm) above which infection by *Eurychasma* was no longer observed in *L. digitata* sporophytes is coincident with the threshold size for the acquisition of the capacity to respond with an oxidative burst to elicitation with alginate oligosaccharides.

However, we show here that protection of *L. digitata* against *L. tomentosoides* was not significantly established until at least 7 days after host elicitation with oligoguluronates and that it persisted for one more week. These observations suggest that secondary, long-term defense mechanisms are induced downstream of the oxidative burst, a build-up of immunity that probably requires the progressive up-regulation of biosynthetic enzymes as well as ultrastructural changes. Thus we postulate that the establishment of resistance involves the synthesis of chemical defenses, which are either toxic to *Laminariocolax* zoospores and/or result in the host cell wall strengthening, providing a barrier against penetration by the pathogen.

Chemical defenses of marine brown algae include secondary metabolites such as terpenoids and polyphenols (Hay and Fenical, 1988, 1992; Steinberg, 1992; Hay and Steinberg, 1992; Cronin and Hay, 1996; Hay, 1996; Targett and Arnold, 1998; Pavia and Toth, 2000), as well as fatty acid-derived compounds such

as C_{11} hydrocarbons (Hay et al., 1998; Schnitzler et al., 1998). Interestingly, accumulation of UV autofluorescent material was observed in the *L. digitata* plantlets elicited with alginate oligosaccharides as well as at the penetration sites of *Laminariocolax* filaments, and we hypothesize that these UV fluorescent compounds correspond to aromatic secondary metabolites such as phlorotannins. It was demonstrated that these defense metabolites are inducible by grazers in brown algae (Pavia and Toth, 2000) and that this can increase resistance to further grazing (Toth and Pavia, 2000). There is still some debate, however, as to the nature of the signals that induce the up-regulation of phlorotannins. In Laminariales, mechanical wounding induces rapid accumulation of these compounds (Hammerstrom et al., 1998). In contrast, in the fucalean *Ascophyllum nodosum*, mechanical wounding did not properly mimic the action of the grazer *Littorina obtusata* regarding the build-up of phlorotannin defenses (Pavia and Toth, 2000). In light of our results, we propose that, besides mechanical wounding, signaling through alginate oligosaccharides may be a cue for the recognition of grazing by marine molluscs.

Among the 11 brown algal orders under investigation here, high levels of constitutive release of hydrogen peroxide were observed in species that feature filamentous erect thalli or microthalli. Such a behavior may constitute a nonenzymatic mechanism for the detoxification of activated oxygen species generated from photosynthesis or respiration (Collén and Davison, 1999). The alginate-induced oxidative response was found in one member of the Ectocarpales (*Pylaiella littoralis*), and in the sporophytes of all of the Desmarestiales and Laminariales tested. Whereas most fucoids and filamentous species, including the gametophytes of Laminariales, were insensitive to challenges with oligoguluronates (at least in terms of the oxidative response.

Based on recent phylogenies of brown algae (Draisma et al., 2001; Rousseau et al., 2001), no clear evolutionary pattern can be drawn from the distribution of the alginate-induced oxidative response in this survey. However, it was found in one member of the Ectocarpales, in the Desmarestiales, and in the Laminariales, i.e., clades that have evolved the most recently. With the exception of *P. littoralis*, the capacity to perform an alginate-induced oxidative burst may also be thought of as a developmentally regulated characteristic of the alginate-rich members of brown algae with complex, foliose, or frondose macrothalli, such as the sporophytes of Laminariales. Compared to the sporophytes, kelp gametophytes are very susceptible to infection by *Eurychasma dicksonii* (Müller et al., 1999) and the protist parasite *Maullinia ectocarpii* (Plasmodiophorales, Maier et al., 2000). The usually short life span and simple morphology of gametophytes may have necessitated a constitutive rather than an inducible defense system. It can not be ruled out, however, that the macrothallic brown algae that do not respond to a challenge with oligoguluronates by a burst of H_2O_2 are probably endowed with other, inducible defense mechanisms or that they possess other responses to oligoalginate challenge. In this respect, the fucoid *Ascophyllum nodosum*, which was

not responsive to oligoalginates (Table 1), was nevertheless recently shown to be capable of expressing inducible defenses against herbivores (Pavia and Toth, 2000).

In conclusion, the capacity to recognize alginate oligosaccharides as defense signals is limited to a few members of brown algae. From a bioenergetic viewpoint, elicitor recognition followed by a massive, yet transient, release of hydrogen peroxide appears as a rather economic (in terms of redox equivalents required for the reduction of oxygen) and finely tuned form of defense signaling. In addition, our study shows that recognition of alginate oligosaccharides and the related oxidative response are involved with natural and induced immunity of kelps against epiphytic bacteria and endophytes. In any case, these elicitors can now be used to explore the biochemical basis of kelp immunity, including identification of the chemical nature of the inducible defenses. In particular, the *Laminaria digitata–Laminariocolax tomentosoides* host–pathogen system employed in this study provides an experimental model to further investigate the regulation mechanisms and the biological activity of phlorotannins in kelps.

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INDUCTION OF PSEUDOPTEROSIN BIOSYNTHESIS IN THE GORGONIAN *Pseudopterogorgia elisabethae*

RENEE S. THORNTON and RUSSELL G. KERR*

Department of Chemistry and Biochemistry
Center for Molecular Biology and Biotechnology
Florida Atlantic University
Boca Raton, Florida 33431-0991, USA

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Abstract—A number of biological and physical stimuli have been evaluated as inducers of pseudopterosin biosynthesis in the sea whip *Pseudopterogorgia elisabethae.* Data indicate that the production of pseudopterosins can be significantly increased in response to high levels of predation by the mollusk *Cyphoma gibbosum* and in response to decreased levels of UV/visible radiation. High levels of feeding by *Chaetodon capistratus* and an artificial wounding of the sea whip did not result in increased pseudopterosin levels.

Key Words—*Pseudopterogorgia elisabethae*, induction, terpene, marine, biosynthesis, pseudopterosin, predation.

INTRODUCTION

The pseudopterosins represent a class of diterpene glycosides produced by the octocoral, *Pseudopterogorgia elisabethae*. As with many natural products, diverse congeners of the pseudopterosins exist and vary with geographic location. Pseudopterosins A–D were isolated from the nonpolar fraction of the extract from *P. elisabethae* collected from Sweetings Cay, Bahamas (Look et al., 1986). Pseudopterosins E and F are major components of *P. elisabethae* from Bermuda, while pseudopterosins G–J are minor components from the same organism. Pseudopterosins K and L have been found in *P. elisabethae* from samples collected at Great Abaco Island in the Bahamas (Roussis et al., 1990). Little is known about the ecological role of the pseudopterosins although, Fenical (personal communication) has demonstrated that these natural products act as fish feeding deterrents.

* To whom correspondence should be addressed. E-mail: rkerr@fau.edu

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The pseudopterosins are antiinflammatory and analgesic agents with potencies superior to that of existing drugs such as indomethacin in mouse ear models (Look et al., 1986). These compounds appear to act by a novel mechanism of action; they are not active against PLA_2, cyclooxygenase, and cytokine release, or as regulators of adhesion molecules. Evidence suggests the pseudopterosins block eicosanoiod release rather than biosynthesis in murine macrophages (Mayer et al., 1998). Purified pseudopterosins are also used commercially in a cosmetic product, which has generated additional interest in these natural products (Rouhi, 1995).

Factors that control the production and modify the concentration of the pseudopterosins in *P. elisabethae* are unknown. In the course of our studies with this gorgonian (Coleman et al., 1999; Coleman and Kerr, 2000), we have observed that the concentration of the pseudopterosins varies among different geographic locations and also among individuals within one reef area. It is not known whether these differences are due to genetic or environmental factors. There is a limited amount of literature regarding the inducibility of terpenes of terrestrial origin; however, much of the data indicates that many of these natural products are inducible. Various groups have demonstrated the inducibility of paclitaxel and other taxoids with methyl jasmonate in cell cultures of *Taxus canadensis*, *T. cuspidate*, and *T. baccata* (Ketchum et al., 1999a,b; Laskaris et al., 1999). In one study (Laskaris et al., 1999), geranylgeranyl diphosphate synthase activity was rapidly induced in response to the addition of methyl jasmonate to *T. baccata* cell suspension cultures. In the grand fir, *Abies grandis*, biosynthesis of monoterpenes, sesquiterpenes, and diterpenes was shown to be inducible by stem wounding at the level of gene activation; an increase in enzyme activity of the respective terpene synthases was also observed (Bohlmann and Croteau, 1999). Herbivore-induced terpene biosynthesis has been demonstrated in maize (Degenhardt and Gershenzon, 2000). Specifically, upon herbivore attack, maize emits a mixture of volatile compounds including an unusual acyclic C_{11} homoterpene.

The goal of the study described herein is to identify factors that modify the concentration of pseudopterosins in the host gorgonian. Developing an understanding of the ecological role of the pseudopterosins, and specifically identifying induction stimuli, may provide valuable information for a controlled production of these compounds.

METHODS AND MATERIALS

Fieldwork was performed at the east end of Grand Bahama Island, at a depth of 12 m. A area 20×30 m^2 that had a dense gorgonian fauna was selected for the study. *P*. elisabethae is difficult to identify according to morphological characteristics; thus, following a tentative visual identification, each gorgonian was sampled and assayed for the presence of the characteristic pseudopterosins by TLC analysis. Clippings of ca. 10–15 cm of each *P. elisabethae* were removed prior to and

following each treatment in order to quantify changes in pseudopterosin content. Individual gorgonians with at least two branches were selected so that separate, intact branches could be removed for both analyses.

Biological Stimuli. To determine the effects of grazing on the inducibility of pseudopterosins, known predators were confined to an area with one *P. elisabethae* as the only food source. Twenty four-eyed butterfly fish, *Chaetodon capistratus*, were collected from the same site as that used for the induction experiment. Strong mesh-type nylon rope nets with 5-mm openings were used to contain two fish around each of 10 *P. elisabethae.* Another 10 nets acted as a control, containing only the gorgonian and no fish. Floats were placed on the top of the nets to keep them afloat, while the base of the nets encircled the substratum of the gorgonian and were secured with plastic ties. In addition, there were two metal rings, with a rigid 50-cm-diam., which did not allow the nets to touch the branches of the colonies.

Another 20 of the tagged gorgonians were used for the flamingo tongue snail experiment. Thirty flamingo tongue snails, *Cyphoma gibbosum*, were collected and a 2-mm hole was drilled into their shell using a dental diamond burr. In this process, we were careful not to harm the snail's mantle. (This procedure was performed with a group of *C. gibbosum* prior to our induction study to confirm that this would not be harmful to the snails.) Three snails per gorgonian were threaded and tied to the main axis of 10 *P. elisabethae* to create an abnormally high level of feeding on the corals. The snails were placed on the main axial tissue of the gorgonian. Every day, observations were made to ensure the snails were still attached and feeding on the corals. Three fishing lines were tied to each of the other 10 *P. elisabethae* for the control group. Both experiments were left in place for a total of five days, and clippings were removed for post treatment analysis.

Physical Stimuli. To determine the effects of physical stimuli on the inducibility of pseudopterosins, 20 *P. elisabethae* were tagged and divided into two groups—treatment and control. The 10 treatment gorgonians were artificially wounded by slashing with dive knives on all major branches over their entire surface twice a day for 5 days. The 10 control organisms were subject to identical conditions but were not slashed with knives. Prior to treatment, a 10-cm clipping was removed from all gorgonians. At the conclusion of the experiment period, a posttreatment sample similar to the pretreatment sample was removed.

Visible and Ultraviolet light. To determine the effect of visible and UV light on the inducibility of pseudopterosins, 10 *P. elisabethae* were subjected to reduced radiation by shading with acrylic sheets. Frames were constructed using PVC pipes with a 2.4-cm diam. Each frame measured 1×1 m, and acrylic sheets were attached to one side of each frame by plastic ties. Five of the gorgonians had acrylic sheets suspended above them that blocked UV radiation. The other frames blocked visible and UV radiation from reaching the other five. Since the PVC frames are neutrally buoyant, four polypropylene ropes were attached to the corners of the

frames, and the other end of the ropes were tied to four concrete blocks. This allowed the frames to be suspended 5–10 cm above the organisms. Pretreatment and posttreatment clippings were removed from each of 10 gorgonians.

Chemical Analysis. Pseudopterosins were extracted and purified from the experimental clippings according to the following protocol. Each organism was cut into 1-cm pieces and lyophilized for a period of 48 hr. Each sample was extracted three times for 24 hr with 40 ml methanol–methylene chloride (1:1) and concentrated to dryness. The crude extract was partitioned between MeOH–H_2O (9:1) and hexanes, and the resulting MeOH–H_2O layer subsequently extracted with CH_2Cl_2. The latter fraction, containing the pseudopterosins, was evaporated to dryness. The weight of the crude pseudopterosin fraction was obtained, and sufficient ethyl acetate was added to each sample to provide uniform concentrations. The individual pseudopterosin extracts were then further purified by normal phase HPLC using a gradient of hexanes–ethyl acetate (60:40) to ethyl acetate over 25 min. A calibration curve was generated by the use of HPLC utilizing pseudopterosin standards so that the concentrations of the pseudopterosins in our test samples could be quantified.

Pseudopterosin C (Ps C) concentrations were reported as a measure of the overall pseudopterosin content. Ps C is the predominant component of this mixture (ca. 75%) and, thus, is indicative of the overall changes occurring. While not reported in detail, the other pseudopterosins exhibited similar changes in concentration to that reported for Ps C.

Statistical Analysis. The response was taken as the difference between the pseudopterosin content of the pretreatment and posttreatment clippings from each organism. For the fish, snail, and artificial wounding experiments, an analysis of variance was performed using one-way ANOVA to determine if any change in the pseudopterosin content was significant. Results of the UV/visible blocking experiments were analyzed by paired comparison *t* tests. The null hypothesis states that there will be no significant difference in the pseudopterosin content between the control and treatment groups.

RESULTS

Certain fish and mollusks have been observed to prey upon *P. elisabethae*. Lasker (1985) reported that the four-eyed butterfly fish, *Chaetodon capistratus*, feeds on the polyps of *Pseudopterogorgia* spp. The flamingo-tongue snail, *Cyphoma gibbosum*, is a gorgonian specialist and has been observed feeding mainly on the axial tissues of gorgonians including *P. elisabethae* (personal observation; Steudler et al., 1977; Vrolijk and Targett, 1992). These predators were, therefore, selected as potential inducers of pseudopterosin biosynthesis.

In the attempted induction by *C. capistratus*, in which a pair was enclosed with a *P. elisabethae* by a net, intense feeding by the fish on the gorgonian was

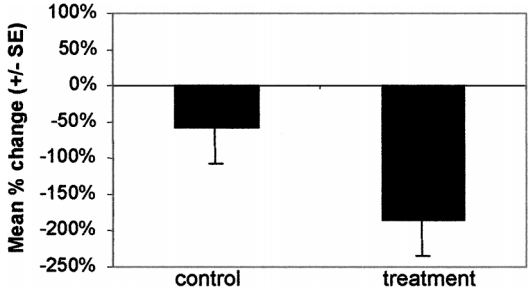


FIG. 1. Effect of elevated *C. capistratus* feeding on pseudopterosin C content. Bars indicate mean $\pm$ SE, $N = 10$.

observed daily. Analysis of the pseudopterosin content of the pre- and posttreatment clippings of the 10 *P. elisabethae* used in this experiment and the 10 control populations indicated that the pseudopterosin content did not significantly change over the treatment period (Figure 1). The one-way ANOVA analysis afforded a P value of 0.558 ($\alpha = 0.05$). The decrease in the pseudopterosins observed in both the control and treatment groups may be a result of decreased health of the corals during this experiment but is not fully understood at this time.

The experiment designed to provide abnormally high levels of feeding by the mollusk *C. gibbosum* resulted in greatly elevated pseudopterosin concentrations (Figure 2). The snails were observed on a daily basis to be feeding on the gorgonian tissue yet this resulted in only a modest amount of damage (Figure 2). ANOVA gave a P-value of 0.002 ($\alpha = 0.05$), indicating the inducibility of pseudopterosin biosynthesis by predation of *C. gibbosum*.

As discussed above, stem wounding has been demonstrated to lead to an induction in terpene biosynthesis in terrestrial systems. It is evident from Figure 3 that the artificial wounding of the gorgonian tissue resulted in only a minimal

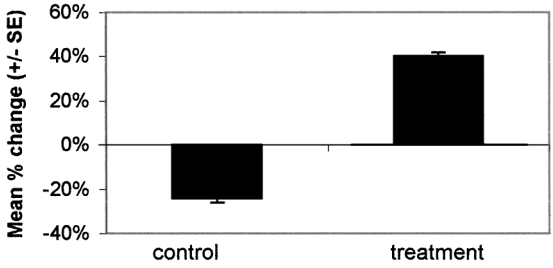


FIG. 2. Effect of *C. gibbosum* feeding on pseudopterosin C content. Bars indicate mean $\pm$ SE, $N = 10$.

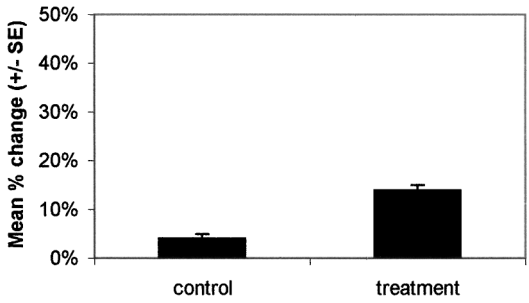


FIG. 3. Effect of rasping of *P. elisabethae* on pseudopterosin C content. Bars indicate mean $\pm$ SE, $N = 10$.

apparent increase in pseudopterosin content. In fact, when the data were subjected to ANOVA, the difference was shown to be not significant.

The effect of decreased visible and UV radiation on pseudopterosin biosynthesis was examined by installing frames with acrylic sheets 5–10 cm above specimens of *P. elisabethae*. The acrylic sheets blocked either UVB or both UVB and visible light from the gorgonians. We were aware that this design allowed some indirect light to reach the corals. The goal of our experiment was not to rigorously exclude all UV or UV/visible light, but only to decrease the amount of radiation significantly. Pre- and posttreatment clippings were removed, and the pseudopterosin content was determined. An analysis of the 10 organisms that were subjected to reduced UV and UV/visible radiation was performed by paired comparison *t* tests. The difference in concentrations of pseudopterosin between the pre- and posttreatment clippings was calculated and the average difference was compared to the null model of zero change. The blocking of UV radiation and both visible and UV radiation increased the pseudopterosin content by approximately 100% in the treatment groups with P values of 0.04 and 0.07, respectively ($\alpha = 0.05$). This is significant, and the null hypothesis was rejected (Figure 4). Interestingly, these results are contrary to those reported by Kinzie (1993) with reef building corals.

DISCUSSION

The overall goal of these experiments was to identify factors responsible for the induction of pseudopterosins in the gorgonian *P. elisabethae.* Our data indicate that the biosynthesis of these natural products can be significantly increased in response to high levels of predation by the mollusk *C. gibbosum* and in response to decreased levels of UV/visible radiation. The induction in response to predation by the mollusk is somewhat comparable to insect-induced terpene biosynthesis in vascular plants. The blocking of UV/visible radiation increased the pseudopterosin

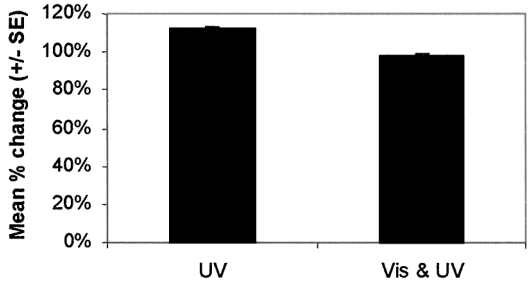


FIG. 4. Effect of blocking UVB and UVB/visible radiation on pseudopterosin C content. Bars indicate mean ± SE, $N = 5$.

content by approximately 100% in the treatment groups. This is intriguing, as the ecological significance is not immediately clear. It seems to suggest the involvement, either directly or indirectly, of a photosynthetic organism in pseudopterosin biosynthesis. Zooxanthellae are well known to be associated with gorgonians and may be involved in this biosynthetic pathway.

To our knowledge, the data with *C. gibbosum* and UV/visible radiation represent the first demonstration of induction of pseudopterosin biosynthesis. The lack of a significant induction in pseudopterosin biosynthesis in response to fish feeding and artificial wounding experiments does not necessarily imply that these stimuli are not inducers (or a mimic of an inducer). Rather, it is conceivable that our experimental protocol did not permit the detection of pseudopterosin induction. Experiments to explore the induction of pseudopterosin biosynthesis more fully are underway.

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COMPARISON OF THE WOUND-ACTIVATED TRANSFORMATION OF CAULERPENYNE BY INVASIVE AND NONINVASIVE *Caulerpa* SPECIES OF THE MEDITERRANEAN

VERENA JUNG,[1] THIERRY THIBAUT,[2] ALEXANDRE MEINESZ,[2] and GEORG POHNERT[1,*]

[1]*Max-Planck-Institut für Chemische Ökologie*
Winzerlaer Str. 10
D-07745 Jena, Germany

[2]*Laboratoire Environnement Marin Littoral*
Université de Nice Sophia-Antipolis
Faculté des Sciences, parc Valrose
06108 Nice cedex 02, France

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Abstract—The invasive green alga, *Caulerpa taxifolia*, that has spread rapidly after its introduction into the Mediterranean and the North American Pacific, reacts to wounding by transforming its major metabolite caulerpenyne (**1**). This wound-activated reaction involves the transformation of the bis-enol acetate moiety of **1**, releasing reactive 1,4-dialdehydes. The ability to perform this transformation is found also in both the noninvasive Mediterranean *C. prolifera* and the invasive *C. racemosa*. Trapping experiments, as well as transformation of the model substrate geranyl acetate, suggest that all three investigated *Caulerpa* spp. rely on esterases that act upon wounding of the algae by subsequently removing the three acetate residues of caulerpenyne. The resulting reactive 1,4-dialdehyde oxytoxin 2 (**9**) can be identified by liquid chromatography–mass spectrometry and is unstable in the wounded tissue. Caulerpenyne transformation occurs rapidly, and severe tissue damage caused degradation of more than 50% of the stored caulerpenyne within 1 min in all three algae. Prevention of the enzymatic reaction before extraction, by shock freezing the tissue with liquid nitrogen, was used for the determination of the caulerpenyne content in intact algae. It gives about twofold higher values compared to an established methanol extraction protocol. The speed and mechanism of the wound-activated transformation, as well as the caulerpenyne content in intact tissue of invasive and noninvasive *Caulerpa* spp., are comparable. Thus, this enzymatic transformation, despite

* To whom correspondence should be addressed. E-mail: Pohnert@ice.mpg.de

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being fast and efficient, is likely not the key for the success of the investigated invasive species.

Key Words—Activated defense, green algae, *Caulerpa*, caulerpenyne, chemical defense, invasive algae, Mediterranean, terpenes, quantification, esterase, 1,4-dialdehydes.

INTRODUCTION

Members of the order Caulerpales are some of the most abundant and widely distributed algae throughout the tropical oceans. This group of siphonous green algae is among the best investigated, both biologically and chemically (Paul and Fenical, 1987; Meyer and Paul, 1992). Growing in reef habitats with intense grazing, these algae have developed efficient chemical defenses that are mainly based on terpenoids. The accidental introduction of *Caulerpa taxifolia* (Vahl) C. Agardh into the Mediterranean in 1984 (Meinesz and Hesse, 1991) and its success in conquering the new habitat (Belsher and Meinesz, 1995; Delgado et al., 1995; Ceccherelli and Cinelli, 1997; Meinesz et al., 2001) has only recently put the focus back on these seaweeds. In addition to its light and temperature tolerance (Garcia et al., 1996; Komatsu et al., 1997) and fast growth (Komatsu et al., 1994; Meinesz et al., 1995), its chemical defense also has facilitated the invasion (Guerriero et al., 1992, 1993; Bellan-Santini et al., 1996).

Recent monitoring revealed that *C. taxifolia* is not the only invasive *Caulerpa* species spreading in the Mediterranean. One introduced variety of *C. racemosa*, *C. racemosa* var. *occidentalis* (J. Agardh) Borgesen, with high temperature tolerance, was identified and has been spreading continuously since the early 1990s (Verlaque et al., 2000). Besides those introduced and invasive species, there exist the indigeneous noninvasive *C. prolifera* (Forsskal) Lamouroux that has been proven to be useful in comparison studies for investigations of the factors determining the invasive potential (Sant et al., 1996).

The role of the sesquiterpene caulerpenyne (**1**) (Figure 1), initially isolated from Mediterranean *C. prolifera* (Amico et al., 1978), in the chemical defense of species of *Caulerpa* has been investigated in numerous studies. Caulerpenyne acts as a feeding deterrent to tropical sea urchins (McConnell et al., 1982) and has been related to the poor food quality that *C. taxifolia* has for Mediterranean sea urchins (Boudouresque et al., 1996). It inhibits the growth of microorganisms, interferes with the development of fertilized sea urchin eggs, and is toxic to larvae and adults of potential herbivores (Paul and Fenical, 1986; Pesando et al., 1996; Lemée et al., 1997; Ricci et al., 1999). On the other hand, studies with herbivorous tropical fishes have shown that caulerpenyne does not defend the plants against these macroherbivores (for an overview see Meyer and Paul, 1992). Several studies have focused also on the caulerpenyne content in various *Caulerpa* species (e.g. Paul and Fenical, 1986; Meyer and Paul, 1992; Gavagnin et al., 1994; Amade and

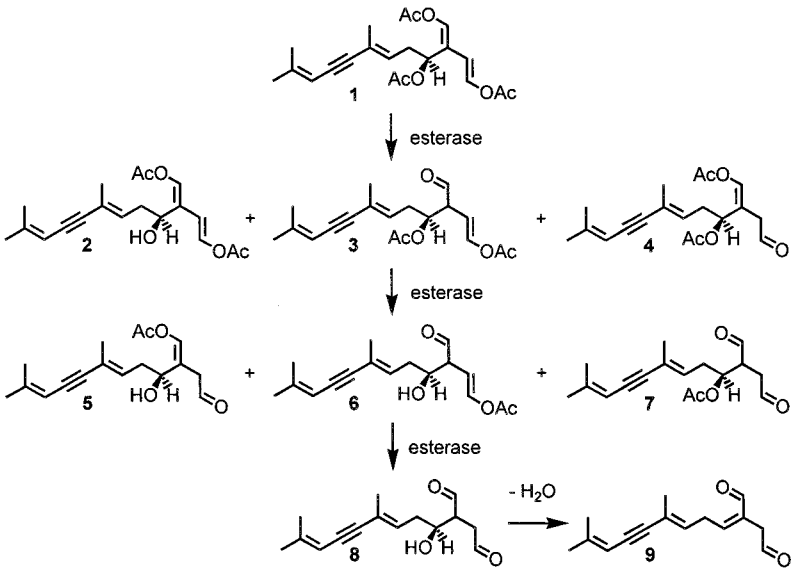


FIG. 1. Stepwise deacetylation of caulerpenyne **1** by esterases from *C. taxifolia*. (**9** = oxytoxin 2; **4** = oxytoxin 1).

Lemée, 1998). Throughout this order, caulerpenyne was found to be the major or one of the major secondary metabolites, the overall content varied depending on species, location, and season. The aquarium derived strain of *C. taxifolia* (Jousson et al., 1998, 2000; Wiedenmann et al., 2001), spreading in the Mediterranean, can contain up to 1.9% of the algal dry weight (DW) of caulerpenyne, but this value depends on season and depth (Amade and Lemée, 1998). In contrast, Pacific *C. racemosa* species collected in Guam contained only 0.2–0.5% DW (Meyer and Paul, 1992). Not only does the caulerpenyne content vary among different species but also within different parts—fronds and stolons—of one individual alga (Meyer and Paul, 1992).

While these investigations have mainly focused on caulerpenyne, recent evidence suggests that the presence of caulerpenyne-derived sesquiterpenes in wounded algal tissue also have to be taken into account. We found that upon mechanical wounding of *C. taxifolia*, caulerpenyne is rapidly transformed into reactive aldehydes (Jung and Pohnert, 2001). This reaction is comparable to the wound-activated conversion of the diterpene halimedatetraacetate into the potent feeding deterrent halimedatrial in algae of the related genus Halimeda (Paul and Van Alstyne, 1992). The identified products from *C. taxifolia* and the use of model substrates suggest an enzymatic transformation as shown in Figure 1 (Jung and Pohnert, 2001).

The resulting aldehydes **4** and **9** from *C. taxifolia* are of the oxytoxin family and have been previously found in defensive secretions and parapods of specialized mollusks feeding on *C. prolifera* (Gavagnin et al., 1994). Under attack, these mollusks secrete a toxic mucus or, if molested further, detach their tail or parapods to distract the enemy and escape. The mucus and the parapods contain the aldehydes **4** and **9** as well as caulerpenyne and are capable of deterring various predators (Cimino et al., 1990; Thibaut et al., 2001). Although directly involved in the defense of slugs against predatory fishes (Gavagnin et al., 1994), the role of these metabolites in a wound-activated algal defense has yet to be evaluated.

Facing the spread of *C. taxifolia* in the Mediterranean, the question arose as to whether the wound-activated transformation of caulerpenyne acting immediately after tissue damage is an important key to success for this species alone or whether this mechanism is universally found among Caulerpales. The major aim of this work was to address this question by performing a comparative study of the production of reactive aldehydes by different *Caulerpa* spp. upon wounding.

Since significant amounts of caulerpenyne are transformed within seconds after wounding (Jung and Pohnert, 2001), a new quantification protocol had to be developed that avoids this reaction during the extraction process. This was achieved by shock freezing the algae with liquid nitrogen prior to extraction, thus suppressing enzyme activity during the work-up. One focus of the present work, therefore, was to provide means to determine and compare the caulerpenyne content in intact tissue of different *Caulerpa* species. We chose three *Caulerpa* species of the Mediterranean: the introduced and invasive aquarium-derived strains *C. taxifolia* and *C. racemosa* and the noninvasive Mediterranean *C. prolifera*. Comparing invasive and noninvasive species from the same habitat will help establish the role of wound-activated caulerpenyne transformation to reactive aldehydes as a potential defensive strategy.

METHODS AND MATERIALS

General. MS-grade solvents were purchased from Riedel de Haen (Seelze, Germany). HPLC-APCI-MS (atmospheric pressure chemical ionization): A Hewlett-Packard HP1100 was equipped with a Macherey-Nagel normal phase Nucleosil 50–7 column (250 $\times$ 4 mm) for LC separation; a Finnigan LCQ with APCI interface was used for detection. High-resolution gas chromatography–mass spectrometry GC-HR-MS was performed on a Micromass MasSpec double-focusing magnetic sector mass spectrometer (geometry EBE) connected to a Hewlett Packard HP6890 II gas chromatograph, equipped with a DB-5 (J&W Scientific) capillary column (30 m $\times$ 0.25 mm, 0.25 μm).

Algal Collection and Cultivation. Samples of 1 kg of the three studied species were collected in the south of France during mid- to late summer when the three species exhibit their maximum growth and biomass. Since it is not possible to

collect the three species from one sampling site, care was taken to select all species from calm water. *C. taxifolia* was collected at Villefranche-sur-Mer (France) in July 2000, at a depth of 5 m. *C. prolifera* was collected at Cap d'Antibes (France) in October 2001, at 1 m, and *C. racemosa* was obtained from Villefranche-sur-Mer also in October 2001 at a depth of 15 m. All algae were transferred to aerated aquaria with 10–30 liters of artificial seawater (Instant Ocean, Aquarium Systems, Mentor, Ohio, USA) for preequilibration before extraction. The aquaria were illuminated with a 14L:10D photoregime and kept at $24 \pm 2^{\circ}$C. Half the water was exchanged monthly. Only intact individuals with a weight of 1–3 g that did not show any sign of mechanical damage or bleaching were randomly selected for extraction.

Extraction and Quantification of Caulerpenyne. For the determination of the caulerpenyne content in intact tissue, enzymatic degradation was prevented by freezing 3 g of the algae (fronds and stolons) with excess liquid nitrogen. The cold material was ground to a powder and treated with 3 ml ethyl acetate containing 1 mg/ml 4-methoxyacetophenone (Amade and Lemée, 1998) as an internal standard. During warm-up to 0°C, grinding was continued and the cold mixture was centrifuged 5 min at 15,000*g* for phase separation. The organic phase (20 μl) was used directly for quantification with normal-phase (NP)-HPLC-MS [diode array detector (DAD), detection wavelength 252 nm]. HPLC gradient was: hexane–ethyl acetate; 1 ml/min: 0%, 12 min 25%, 18 min 35%, 18 min 0%, 25 min 0% ethyl acetate. Reextraction of the algal residues with 2×3 ml of ethyl acetate showed an extraction efficacy of $>95\%$ caulerpenyne in the first step. For comparison, other extraction methods were tested on the frozen algal powder. One method used 3 ml of methanol containing 1 mg/ml 4-methoxyacetophenone instead of ethyl acetate as solvent. 20 μl of the alcoholic solution were analyzed directly with NP-HPLC-MS. Repeated extractions ($3\times$) showed poor and unreliable extraction efficacy for the first extraction step (78–91%). The other method tested the extraction protocol of Bligh and Dyer (1959) using a ternary mixture of water, chloroform, and methanol as solvent. To 3 g ground algal material obtained after freezing with liquid nitrogen, 1.25 ml chloroform and 2.5 ml methanol containing 3 mg 4-methoxyacetophenone were added and shaken for 2 min. For phase separation, 1.25 ml water and 1.25 ml chloroform were added. After centrifugation, 20 μl of the organic phase were applied to NP-HPLC-MS. While the extraction efficacy was around 95%, the caulerpenyne purification was complicated by elevated amounts of extracted lipids.

Purity of the caulerpenyne signal was monitored by using DAD and MS detection (APCI). For quantification, a linear calibration curve obtained with purified (column chromatography with SiO_2 and Florisil) caulerpenyne was used. The response was linear from 0.1 to 10 μg caulerpenyne ($P < 0.001$, $R = 0.99$, $N = 3$).

Transformation Experiments. For measurements at $t = 0$, the algal material was shock-frozen with liquid nitrogen before extraction as described above. Injury

was simulated by manually grinding 2–3 g of the algae for 30 sec to homogeneity. The homogenate was shock-frozen after defined intervals to prevent further enzymatic degradation and was then extracted with ethyl acetate (1 ml/g algal fresh weight) containing the internal standard. LC quantification was performed as described above. For the relative quantification of oxytoxin 2 (**9**), the APCI MS ion at $m/z = 231$ (Jung and Pohnert, 2001) was monitored and compared to the same ion of caulerpenyne. The identity of oxytoxin 2 (**9**) in the fresh extracts of wounded algae was verified by GC-HR-MS: 230.1305; calculated: 230.1307 [$C_{15}H_{18}O_2$]; EI-MS (70eV; m/z (%): 230(100), 215(42), 201(15), 187(59), 159(45), 143(15), 131(17), 115(14).

Derivatization of Crude Algal Extracts. Trapping experiments using 4 g algal material and 1 ml of a saturated ethanolic 2,4-dinitrophenylhydrazine (DNPH) solution were performed as described previously (Jung and Pohnert, 2001).

Enzymatic Transformation of Model Substrates. 5 g of the algae were carefully ground with 20 mg of geranyl acetate in 5 ml seawater. After defined time spans, 500 μl samples were taken and extracted with 100 μl dichlormethane. GC-MS was used for the identification and quantification of geraniol and geranyl acetate, respectively.

RESULTS

Caulerpenyne Content in Intact Algae. We compared the caulerpenyne content in the three *Caulerpa* species selected. These were frozen with liquid nitrogen before extraction to prevent enzymatic caulerpenyne degradation (Figure 2). While *C. taxifolia* and *C. prolifera* contain almost the same amount of caulerpenyne,

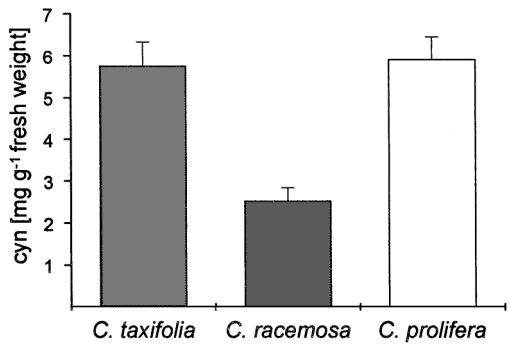


FIG. 2. Mean values ($\pm$ SE; $N = 3$) of caulerpenyne (cyn) per gram of algal fresh weight in intact tissue of three different *Caulerpa* spp.; intact algal material was shock-frozen, ground, and extracted with 1 ml/g alga ethyl acetate containing 1 mg/ml 4-methoxyacetophenone as internal standard.

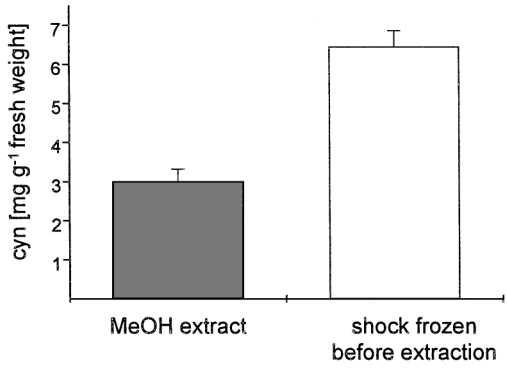


FIG. 3. Determination of caulerpenyne (cyn) content in *C. taxifolia* grown in one aquarium following two different extraction protocols. An established method (Amade and Lemée, 1998) that involves overlaying of the algae with methanol and freezing the set-up overnight is compared with the protocol introduced here. Extracting algae that have been shock-frozen prior to grinding with ethyl acetate increases the determined amount twofold. Mean values ($\pm$ SE; $N = 10$) of caulerpenyne are given per gram of algal fresh weight.

6 mg/g of algal fresh weight, the caulerpenyne content in *C. racemosa* was only half this value.

Caulerpenyne from *C. taxifolia* was quantified with two different extraction methods using algae that were equilibrated in one aquarium for 10 months (Figure 3). Following the protocol of Amade and Lemée (1998) involving the transfer of rinsed algae into methanol (10 ml methanol per gram wet alga), which was then frozen overnight, an overall content of 3 ± 0.31 mg caulerpenyne per gram algal fresh weight was determined. Freezing the algae with liquid nitrogen prior to grinding and extraction with ethyl acetate led to an overall content of 6.44 ± 0.41 mg caulerpenyne per gram algal fresh weight, which was twice the amount found with the former method. Comparable amounts of caulerpenyne were also found after extraction of the shock-frozen algae with a solvent mixture according to Bligh and Dyer (1959). In contrast, extraction of the frozen material with methanol led to an underestimation of caulerpenyne in the intact tissue.

Caulerpenyne Transformation by Wounded Algae. Wounding was simulated by manually grinding the algae to homogeneity, and the reaction was stopped by shock-freezing the wounded tissue with liquid nitrogen after certain intervals before extraction. The wound-activated caulerpenyne degradation in the three *Caulerpa* species was compared (Figure 4). In all three species, the caulerpenyne content was significantly reduced within 1 min after wounding. Differences occurred in the initial caulerpenyne content and the speed of degradation in the first minutes. A reduction to 50% of the amount of caulerpenyne after 1 min was found in

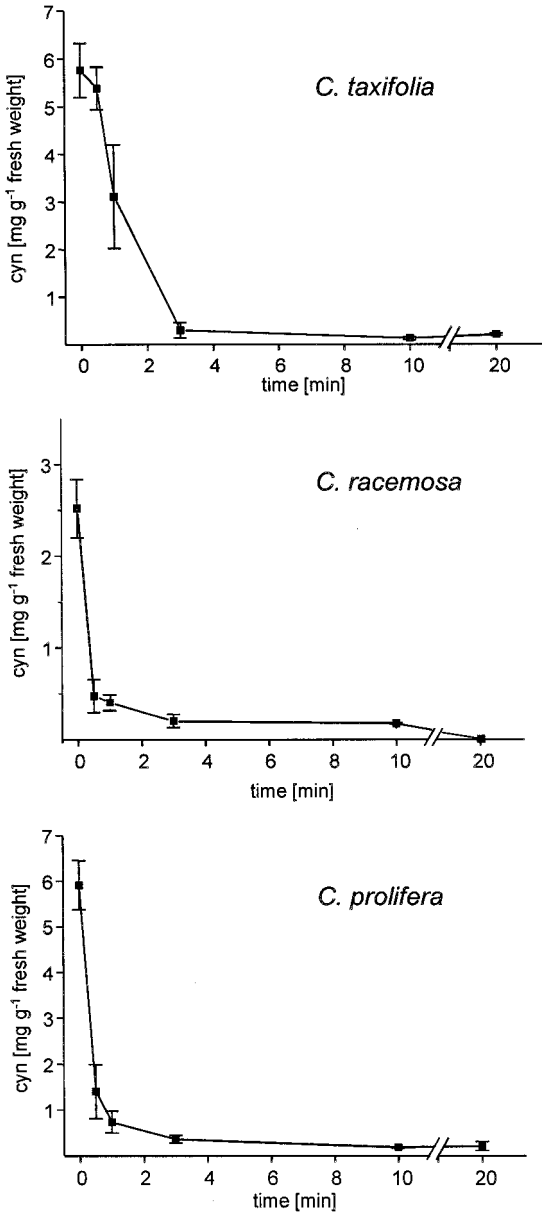


FIG. 4. Wound-activated caulerpenyne (cyn) degradation in three different *Caulerpa* spp.; the dots indicate mean values (± SE; $N = 3$) of caulerpenyne content per gram of algal fresh weight in wounded algae shock-frozen after defined intervals and extracted with ethyl acetate.

C. taxifolia. Although the initial caulerpenyne content found in intact *C. racemosa* (Figure 4) is only half of that in intact *C. taxifolia*, the ratio of caulerpenyne being transformed within the first minute after wounding is comparable. In *C. prolifera*, the most pronounced wound-activated transformation was observed. One minute after wounding, the caulerpenyne content in *C. prolifera* was reduced to 11% of the initial value. A problem that arose during these experiments concerned the polymerization of wounded algal material. In coenocytic green algae, the formation of a wound plug, which prevents loss of protoplasm after wounding, is essential to the survival of these plants (Dreher et al., 1982). This polymer formation occurs after disruption of the cell wall, so that 20 min after wounding further extraction of caulerpenyne and caulerpenyne derived products was hindered.

The ratio of the end product (oxytoxin 2) to the starting product (caulerpenyne) determined in HPLC-MS measurements exhibits a dynamic course. In the first 3 min, the relative amount of oxytoxin 2 (**9**) increases, but after 3 min, when caulerpenyne degradation slows, a decrease of this compound is detectable. It is likely that the reactive 1,4-dialdehyde oxytoxin 2 (**9**) undergoes further reaction with, for example, nucleophiles from the wounded algal tissue. The identity of oxytoxin 2 (**9**) was verified directly after shock-freezing and extracting wounded algal tissue of *C. prolifera* by HR-MS.

Trapping of Reactive Aldehydes. The detection and identification of aldehydes resulting from the wound-activated transformation of caulerpenyne was difficult because of their reactivity and instability. While the amount of oxytoxin 2 (**9**) as the end product shows a transient character, the detection of the intermediates **2–8** is only possible after trapping, followed by concentration. If 2,4-dinitrophenylhydrazine (DNPH) is used as a trapping reagent, the labile intermediate aldehydes react with the reagent to form stable hydrazones that can be concentrated and be submitted to HPLC-UV and HPLC-MS analysis (Jung and Pohnert, 2001). Trapping experiments with DNPH on wounded *C. prolifera* resulted in the detection of a complex series of closely related hydrazones, bearing one or two derivatized aldehydic groups. Their characteristic fragmentation patterns, molecular ions, chromatographic behavior as well as the comparison with hydrazones derived from wounded *C. taxifolia*, were in agreement with derivatives of **3–8** and **9** after elimination of water (Jung and Pohnert, 2001). The stepwise cleavage of acetyl residues and the rapid activation after disruption of cell compartmentation suggests the involvement of an enzymatic mechanism.

Esterase Activity in Wounded Algae. The action of an enzymatic esterase mechanism was verified by using model substrates. The sesquiterpene farnesyl acetate and, to challenge substrate specificity, the monoterpene geranyl acetate were applied to wounded algal tissue. Indeed, esterases transformed these acetates into their corresponding alcohols. GC-MS analysis allowed monitoring of the formation of the deacetylated alcohols farnesol and geraniol. This mechanism, previously shown to be active in *C. taxifolia* (Jung and Pohnert, 2001), is also found

in *C. prolifera* and *C. racemosa* after wounding. Amounts of up to 4 mg acetate per gram algal fresh weight were transformed into the corresponding alcohols within 1 hr by all three *Caulerpa* spp. Again, as with the wound-activated transformation of caulerpenyne itself, *C. prolifera* proved to be the most efficient alga in the transformation of the model substrate.

DISCUSSION

We recently reported that the invasive alga *C. taxifolia* transforms its major secondary metabolite, the sesquiterpene caulerpenyne, into highly reactive aldehydes (Jung and Pohnert, 2001). The current study was undertaken to determine if this wound-activated cleavage of acetyl moieties from caulerpenyne (Figure 1) is restricted to the invasive *C. taxifolia*, or if it is a more general feature of invasive and noninvasive *Caulerpa* spp. As shown with trapping experiments and the kinetic analysis (Figure 4), all three species investigated react to wounding with rapid transformation of caulerpenyne into a mixture of reactive and partly unstable aldehydes.

The rapid spread of *C. taxifolia* since its introduction into the Mediterranean (Meinesz et al., 2001), as well as after its first detection in California (Kaiser, 2000), has been attributed to factors such as fast growth and high temperature and substrate tolerance, as well as to an efficient chemical defense (Bellan-Santini et al., 1996). The results presented here show that the caulerpenyne content is comparable in the invasive *C. taxifolia* and the noninvasive *C. prolifera*, but is lower in the invasive *C. racemosa* (Figure 2). The caulerpenyne content of this variety of the latter species was investigated for the first time. The values given here have to be interpreted carefully, since earlier studies (Amade and Lemée, 1998) indicated that the caulerpenyne content in *C. taxifolia* can vary depending on season and depth. A variation in caulerpenyne content was also found in different plant parts (Amade and Lemée, 1998; Meyer and Paul, 1992). For comparison, in this study we collected all algae from calm water and extracted whole plants after preequilibration in aquaria. A careful season-dependent reinvestigation of different *Caulerpa* spp. in selected locations using the new quantification method introduced here will be required for a detailed survey.

The results, however suggest that the difference in the overall caulerpenyne content in intact tissue is not substantially higher in invasive species. The ability to transform this metabolite, after tissue disruption, into reactive aldehydes occurs in the invasive *C. taxifolia* as well as in the noninvasive Mediterranean *C. prolifera*. This noninvasive species transforms caulerpenyne even faster than *C. taxifolia* (Figure 4). In addition, LC-MS characterization and trapping experiments of the transformation products show a similar product spectrum in both algae. *C. racemosa* also exhibits activated transformation of caulerpenyne but contains

a lower overall content of this metabolite compared to either of the investigated species (Figure 2). All these results suggest that the ability of an enzymatic caulerpenyne transformation to reactive aldehydes is not the major factor determining the invasive potential of Caulerpales.

Wound-activated transformation of caulerpenyne consists of the subsequent cleavage of the three acetyl groups present in the molecule. Previous findings using geranyl and farnesyl acetate as model substrates indicate that after wounding, esterases from *C. taxifolia* can cleave these groups with remarkable efficacy (Jung and Pohnert, 2001). While transformation of the model substrates leads to geraniol and farnesol, respectively, the bis-enol acetate moiety of caulerpenyne is cleaved into enols that immediately tautomerize to their corresponding aldehydes. Trapping experiments with DNPH, as well as the LC-MS investigations performed in this study, show that in both *C. taxifolia* and *C. prolifera*, intermediates towards **9** (Figure 1) are detectable. Thus, in both algae, and most likely also in *C. racemosa* that degrades caulerpenyne with similar kinetics, a stepwise transformation of caulerpenyne is observed. The similarity of transformation mechanisms is further supported by the fact that all three algae accept geranyl acetate as a substrate. The kinetic experiments (Figure 4) are in accordance with the observation that the transformation of geranyl acetate is more efficient in *C. prolifera* compared to the other investigated species. Our experiments show that the end product, after cleavage of all three acetyl functions and elimination of water, oxytoxin 2 (**9**) does not accumulate in the wounded algal tissue. Oxytoxin 2 (**9**) can be detected by LC–MS and GC-MS when freshly wounded algae are extracted, and its relative amount compared to caulerpenyne in the algal tissue reaches a maximum 2–3 min after wounding. If not continuously produced, the amount decreases rapidly, presumably by adduct formation and copolymerization. The inherent instability of oxytoxin 2 (**9**) is the likely reason that this molecule was not detected in the numerous previous studies on the secondary metabolites of *Caulerpa* spp. In contrast, the more stable intermediate oxytoxin 1 (**4**) was reported in a detailed analysis of minor metabolites from *C. taxifolia* (Guerriero et al., 1992). Remarkably, the aldehydes oxytoxin 1 (**4**) and oxytoxin 2 (**9**) have been detected previously in the secretion and mucus of sacoglossan herbivores that live in close association with *C. prolifera* (Cimino et al., 1990; Gavagnin et al., 1994). Despite the instability of oxytoxin 2 (**9**), which could not be purified by chromatography, these mollusks are able to accumulate the deacetylated aldehydes in their tissue (Cimino et al., 1990). Whether the mollusks rely on esterases from *Caulerpa* spp. for the transformation of caulerpenyne or utilize their own enzymes will need to be explored.

In algae of the family Caulerpaceae and Udoteaceae, terpenoid metabolites with bis-enol acetate structures are widespread, and several aldehydic analogs that can formally arise from the transformation of these acetylated precursors have been detected (Paul and Fenical, 1987). The first observed example of an activated chemical defense in the marine environment was reported from *Halimeda*

spp. (Udoteaceae), which transforms the bis-enol acetate halimedatetraacetate, presumably via deacetylation, to the more active halimedatrial (Paul and Van Alstyne, 1992). In this case, the unique structure of the postulated intermediate 1, 4-dialdehyde allows an intramolecular enzyme-assisted cyclization to the more stable halimedatrial (Paul and Van Alstyne, 1992). In the light of these studies and our results, a more detailed investigation of the biotransformation of the bis-enol acetates by these green algae is required for a full understanding of wound-activated transformation of their secondary metabolites.

Indirect evidence for the wider distribution of a wound-activated transformation of secondary metabolites was recently given by Cetrulo and Hay (2000), who submitted the extracts of 40 seaweeds to a TLC comparison of the metabolic profile before and after wounding. In both tested *Caulerpa* species (*C. mexicana* and *C. prolifera*), they observed the degradation of a not-further-characterized metabolite 30 sec after wounding. Nevertheless, they did not find any activated chemical defense in experiments where extracts of the wounded algal tissue were coated onto a palatable seagrass. In the light of our findings, these bioassays might not encompass a wound-activated defense due to the instability of the caulerpenyne-derived aldehydes that could degrade during the food impregnation or the assay period. In a comprehensive study of the chemical defense of tropical Caulerpales, Paul and Fenical (1986) tested a multitude of bis-enol acetates and corresponding aldehydes from algal extracts. They observed that, in general, compounds possessing one or more aldehyde functional groups tend to have higher biological activities in antimicrobial-, fish feeding-, sea urchin egg cleavage assays and toxicity tests on larval and adult stages of potential herbivores. These results indicate that it is likely for caulerpenyne deacetylation products shown in Figure 1 to exhibit increased effects against herbivores and pathogens compared to caulerpenyne. Due to the observed instability of the aldehydes, a careful experimental protocol for bioassays will be required, as some of the standard methods that involve incorporation of extracts or secondary metabolites in an artificial diet are not sufficient.

These bioassays, as well as the activity assays of caulerpenyne itself, have to take into account that the total caulerpenyne content in previous studies may have been significantly underestimated. Since transformation of more than 50% of the initial caulerpenyne content was observed in all species investigated within only 1 min after tissue disruption (Figure 4), even in standard solvent extraction procedures caulerpenyne transformation cannot be excluded. Our improved method involves shock-freezing of the intact algal tissue with liquid nitrogen before extraction. Ethyl acetate extracts more than 95% of the caulerpenyne from the frozen algal powder in one step with high reproducibility and allows fast and direct GC and LC analysis not only of caulerpenyne but also of its transformation products. Thus, this solvent proved superior compared to methanol or the solvent mixture of Bligh and Dyer (1959), where poor extraction success was observed and the work-up procedure did not allow the detection of labile caulerpenyne derived

aldehydes. Using the liquid nitrogen ethyl acetate procedure, an underestimation of caulerpenyne in the intact algae due to enzymatic transformations is prevented, and compared to a standard methanolic extraction procedure (Amade and Lemée, 1998), we recovered about twice as much caulerpenyne from *C. taxifolia* (Figure 3).

In summary, we provide the basis for a detailed reexamination of the defensive chemistry of *Caulerpa* species that has to take into account the dynamic effects observed after wounding of the tissue, resulting in reactive transformation products of caulerpenyne.

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ROLE OF SEMIOCHEMICALS IN MATE LOCATION BY PARASITIC SEA LOUSE, *Lepeophtheirus salmonis*

ANNA INGVARSDÓTTIR,[1] MICHAEL A. BIRKETT,[2] IAN DUCE,[3] WILLIAM MORDUE,[1] JOHN A. PICKETT,[2] LESTER J. WADHAMS,[2] and A. JENNIFER MORDUE (LUNTZ)[1,*]

[1]*Department of Zoology*
University of Aberdeen
Tillydrone Avenue
Aberdeen AB24 2TZ, United Kingdom
[2]*Biological Chemistry Division, IACR-Rothamsted*
Harpenden, Herts AL5 2JQ, United Kingdom
[3]*School of Life and Environmental Sciences*
University of Nottingham
University Park, Nottingham NG7 2RD, United Kingdom

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Abstract—The role of olfaction and diffusible pheromones in mate location behavior of sea lice, *Lepeophtheirus salmonis*, was assessed with Y-tube behavioral bioassays. The pheromone "emitting" animals were located in a chamber in one arm of a Y-tube arena, with artificial seawater flowing through both arms. Adult male sea lice displayed both activation and directional responses to seawater conditioned with preadult II virgin females, but were only activated by mated adult female conditioned water. Further, when males were given the choice of preadult II virgin females or mated adult females, a significant number of males chose the arm with the preadult II virgin females. Adult males showed activation responses when presented with water conditioned with adult males but were not attracted to them. When presented with adult males, preadult II virgin females showed only directional responses, but not activation responses. Preadult II virgin female conditioned water was extracted using solid-phase extraction (SPE) protocols pioneered for semiochemical isolation. Adult male sea lice showed significant directional responses to the preadult II virgin female SPE extract. Distillation under vacuum was performed on the extract to give a distillate comprising components with a molecular weight range and physical properties comparable to those of compounds utilized as volatile semiochemicals by terrestrial organisms and a residue comprising components with higher molecular weight range comparable to those utilized as involatile semiochemicals. Adult males were found to be both significantly activated and attracted to

* To whom correspondence should be addressed. E-mail: a.j.mordue@abdn.ac.uk

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the distillate, but not to the residue. This research provides evidence that small, lipophilic organic molecules are used by sea lice as sex pheromone signals to locate a member of the opposite sex.

Key Words—Copepoda, sea lice, salmon, behavior, mate location, mate recognition, chemoreception, chemical ecology, semiochemicals, chemotaxis.

INTRODUCTION

Sea lice, *Lepeophtheirus salmonis*, (Copepoda: Caligidae) are ectoparasites on wild and farmed salmonids, with Atlantic salmon (*Salmo salar* L.) as their main host. Sea lice have a huge financial impact on the salmon farming industry due to the damage they inflict. The industry is largely dependent on the use of veterinary medicines for their control. Environmental concerns and the development of resistance both highlight the need for alternative approaches to sea louse control and for the development of integrated pest management strategies. The use of semiochemicals is one such approach being explored.

After the identification of the first moth sex pheromone, it was suggested that synthetic pheromones could act as efficient communication disruptants and would have high potential as components of integrated pest management (Wright, 1965; Howse et al., 1996; Pickett et al., 1997). Mating communication has been characterized in many insects, and semiochemicals have been used successfully in pest control, in particular the sex pheromones of Lepidoptera and aggregation pheromones of Coleoptera (Howse et al., 1996; Pickett et al., 1997). In aquaculture, studies of sea louse chemical ecology, particularly pheromones and other semiochemicals that influence louse behavior, could provide alternative pest control methods to the use of veterinary medicines.

In contrast to other reproductive features, little is known on the role of chemical cues in mating in copepods, although their significance has been recognized for more than 30 years. Existing evidence suggests that chemical communication in the Copepoda is as complex as described for terrestrial insects. Chemical cues often mediate courtship and mating in aquatic organisms (Katona, 1973; Miller, 1989; Zimmer-Faust, 1991; Ritchie et al., 1996; Doall et al., 1998; Frey et al., 1998; Hull et al., 1998; Kelly et al., 1998; Strickler, 1998; Tsuda and Miller, 1998; Weissburg et al., 1998; Yen et al., 1998), and generally females produce pheromones that attract males and affect their behavior. However, no molecules have been characterized that definitely function as sex pheromones in aquatic organisms. Furthermore, the transmission and reception signals used are not known in any aquatic animal (Snell and Nacionales, 1990; Snell and Morris, 1993).

Semiochemicals form important alternatives to pesticide control by luring, trapping, and disrupting the behavior of insects (Vale et al., 1988; Howse et al., 1996). Kairomones and allomones, i.e., host odor cues or host-masking chemicals,

attract/repel different species respectively, and sex/aggregation pheromones attract the same species (Brown et al., 1970; Law and Regnier, 1971; Blum, 1996). In both marine and terrestrial systems, nonvolatile, hydrophilic semiochemicals detected by contact or gustatory neurophysiological mechanisms are involved in recognition of mates, hosts, or other food sources, and the expectation might be that these would account for the majority of marine interactions. However, processes involving low-molecular-weight lipophilic semiochemicals also exist in marine as well as in terrestrial systems, and in some cases can predominate (Pohnert and Boland, 1997; Ingvarsdóttir et al., 2002). Such compounds possess sufficient solubility for transport in an aqueous medium, which is analogous to transport by means of volatility in air for the terrestrial systems.

The objective of this study was to investigate the role of semiochemicals in sea louse mate recognition and to test the hypothesis that small, lipophilic organic molecules are involved, with physical properties similar to those mediating host location by sea lice (Ingvarsdóttir et al., 2002) and in the chemical ecology of terrestrial organisms. Such pheromones would have potential in integrated sea louse management strategies, for example, in the development of effective traps for sea louse monitoring and control. This would reduce the input of veterinary medicines in aquaculture and provide control programs with intrinsically low environmental impact.

METHODS AND MATERIALS

Sea Lice. Late developmental stages of sea lice (*Lepeophtheirus salmonis*), i.e., adult males (AM), adult females (AF), and preadult II virgin females (PF), were collected from salmon farms on the west coast of Scotland. The lice were kept in fresh seawater from the collection site, on ice, during transport to the University of Aberdeen aquarium. The lice were maintained separately as males and females in the absence of hosts in 25-liter tanks at Aberdeen University at 12°C in artificial aerated seawater of 32‰ salinity and were used from 12 hr to 7 days after collection. Lice normally were observed to show less activity and higher mortality rates after more than 7 days off fish, and healthy batches did not show significant changes in behavioral bioassays within the 7 days after collection (Ingvarsdóttir unpublished).

Y-Tube Assays. A Y-tube arena was situated on top of a light box, and a camera mounted above the arena and linked to a VCR traced the behavior of the lice in the arena. The movements of the lice were recorded on videotape. Y-tube bioassays allowed the sea lice to exhibit a preference for different stimuli compared with seawater controls and also allowed measurement of their activation responses to different stimuli (Ingvarsdóttir et al., 2002). The glass Y tubes had a 16-mm-diam. bore. The arms were 14 cm and the main leg 15.5 cm long (Figure 1). Water flowed through each arm from a reservoir at 30 ml/min. Inlets and outlets of the Y tube

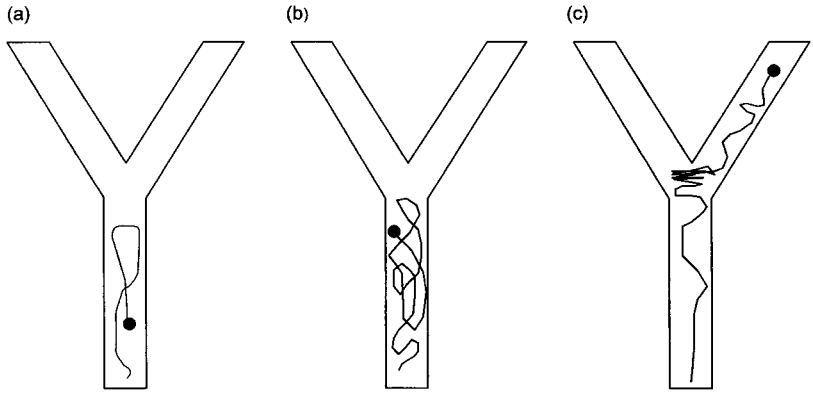


FIG. 1. Diagrammatic representations of Y-tube arena and typical louse activity patterns taken from videotape for activation and taxis behavior of adult male sea lice. Activation was recorded as (a) low activity with distance moved less than twice the length of the base of the Y-tube and (b,c) high activity categories, with distance moved more than twice the length of the base of the Y-tube or choice of arm made. Taxis was recorded as the number of lice entering the odor arm vs the number of lice entering the control arm (redrawn from Ingvarsdóttir et al., 2002).

were made of glass pipets fixed into plastic tops. The tops fitted over the openings on the arms and leg to make watertight seals. There was a clear demarcation of the flow down each arm, and a mixing of the water from each arm in the main leg of the apparatus was demonstrated with food dyes. In double control runs, artificial seawater was introduced into both arms of the Y tube.

The Y-tube experiments were set up in two different ways. Either six individuals of a specific sex and developmental stage (PF, AF, or AM) were placed in a chamber in one arm of the Y-tube and an empty chamber placed in the other arm, with seawater flowing through the chambers in both arms, or previously collected louse-conditioned water (see below) was introduced into one arm while seawater alone was introduced into the other arm prior to introduction of a sea louse. The arm containing the stimulus source was changed regularly, with washing in between, to ensure that there was no positional bias. The chamber restraining the animals to one arm of the Y tube was made up of two pieces of 1-cm Perspex tube inserted into the top of the arm with 200-μm nylon mesh dividing the first chamber from the arm, and another piece of 200-μm mesh between the tubes to keep the animals within the chamber, ensuring water flow over the animals. A single sea louse was introduced into the base of the main leg of the Y tube and allowed 10 mins to respond, typically by moving up the main leg and into one of the arms. Using the Y-tube assay, both overall activity and directional responses were measured in adult male sea lice and preadult II virgin females. Sea louse activation was defined by the degree of movement within the Y tube. Behavior was divided

into two categories, low (Figure 1a) and high (Figure 1b,c) activity (Ingvarsdóttir et al., 2002). Frequencies of intermediate categories between Figure 1a and 1b were low but were defined as less (low) or more (high) than twice the length of the leg of the Y tube. Movement into either arm, regardless of stimulus, was included in the high activity level (Figure 1c). For overall activity levels, the numbers of individuals in low and high activity categories in the presence of different stimuli were compared to those observed with seawater alone. Directional responses (taxis) were measured by the movement of the louse up the main leg and into one of the arms. The number of individuals choosing the stimulus arm instead of the control arm within the 10 min test period were compared with those of seawater controls in both arms. Statistical differences were assessed by χ^2 analysis.

Sea Louse Conditioned Water. Seawater conditioned with preadult II virgin female (PF), adult mated female (AF), and adult male (AM) was prepared by placing six individuals of a specific developmental stage and sex into chambers (as described above) at the top of both arms of a Y tube. Seawater from a reservoir flowed through the chambers at 30 ml/min and was collected in a glass beaker from the outlet of the Y tube. Seawater running through empty chambers divided by mesh was used as a control. Extracts reconstituted for behavioral bioassays were made up to the original concentration with artificial seawater.

Solid-Phase Extraction and Vacuum Distillation. A portable closed solid-phase extraction (SPE) system was used for extracting potential semiochemicals from PF conditioned water (5.17 liters). Artificial seawater (5.0 l) was used as control. The SPE columns [International Sorbent Technology (IST) Limited, Hengoed, Glamorgan, UK] consisted of 6-ml glass cartridges containing layered phases [C_2 (500 mg) over ENV+ (200 mg)]. The extractions were performed using a VacMaster-10 SPE manifold (IST). The SPE cartridges were conditioned prior to extraction with 2 ml HPLC grade methanol, followed by displacement with distilled water (2 ml). Following completion of extraction, interference analytes remaining on the cartridges were removed with distilled water (2 ml). The contents of the cartridges were then extracted with distilled ethanol (2 ml). SPE extracts were separated into two fractions by distillation under vacuum (0.04 torr) for 24 hr at 25°C as described previously (Pickett and Griffiths, 1980). The vacuum distillate comprised components with a molecular weight range comparable to those utilized as volatile semiochemicals by terrestrial organisms, while the residue arising from the vacuum distillation comprised components with insufficient or no volatility. For bioassays, the fractions were redissolved in ethanol and made up to the original extraction volume by adding artificial seawater.

RESULTS

Activation Responses of Sea Lice to Mating Stimuli in Y-Tube Experiments. The activation responses of adult male (AM) and preadult II virgin female (PF)

TABLE 1. ACTIVATION RESPONSE OF SEA LICE TO MATING STIMULI IN Y-TUBE ARENA

Test animal[a]	Odor source	Activity		*N*	*P*
		Low[b] (%)	High[c] (%)		
AM	Seawater control	60.2	39.8	83	
AM	PF conditioned water	25.5	74.5	47	<0.001
AM	PF SPE extract	39.4	60.6	33	<0.05
AM	PF SPE extract: vacuum distillate	29.4	70.6	17	<0.05
AM	PF SPE extract: distillation residue	65.0	35.0	20	NS
AM	AF	40.0	60.0	45	<0.05
AM	PF and AF (in separate arms)	13.3	86.7	30	<0.001
AM	AM	41.7	58.3	48	<0.05
PF	Seawater control	63.3	36.7	30	
PF	AM	58.3	41.7	36	NS

[a] AM = Adult male, PF = Preadult II virgin female, AF = Adult mated female.
[b] Defined as category a in Figure 1.
[c] Defined as categories b and c in Figure 1.

sea lice in the Y-tube bioassay to stimuli derived from the same or different sexes and developmental stages are shown in Table 1. Sixty percent of the AM and PF showed low activity when clean artificial seawater was presented in both arms of the Y tube. In the presence of PF conditioned water, a highly significant increase in adult male activity was observed ($P < 0.001$). Similar results were obtained for PF SPE extract of the conditioned water ($P < 0.05$), and when fractions of the PF extract prepared by vacuum distillation were tested, a significant increase in activity was found only with the vacuum distillate, i.e., the low-molecular-weight fraction ($P < 0.05$). The AM showed increased activity in response to both adult mated females (AF) ($P < 0.05$), and the combined stimulus of AF and PF ($P < 0.001$) presented in separate arms of the Y tube. Further, AM were found to demonstrate a significant activation response in the presence of other males ($P < 0.05$). However, there was no increase in activity, in comparison to seawater controls, for PF to AM.

Directional Responses (Upstream Positive Rheotaxis) of Sea Lice to Mating Stimuli in Y-Tube Experiments. In seawater controls, sea lice showed no particular preference for either arm (Figures 2 and 3). Attraction of AM to PF was clearly demonstrated in the Y-tube experiments (Figure 2). The PF, PF SPE extract, and the PF vacuum distillate of the SPE extract increased the number of AM lice moving to the stimuli, compared to the control arm ($P < 0.001$, <0.05 and <0.05, respectively) (Figure 2). No attraction of AM to the residue arising from the vacuum distillation of the PF SPE extract was observed (Figure 2). Very few males responded directionally to the vacuum distillation residue of the PF extract, and it was not possible to perform χ^2 analysis on the results due to the low numbers. The AM were activated by both AF and AM but did not show significant directional

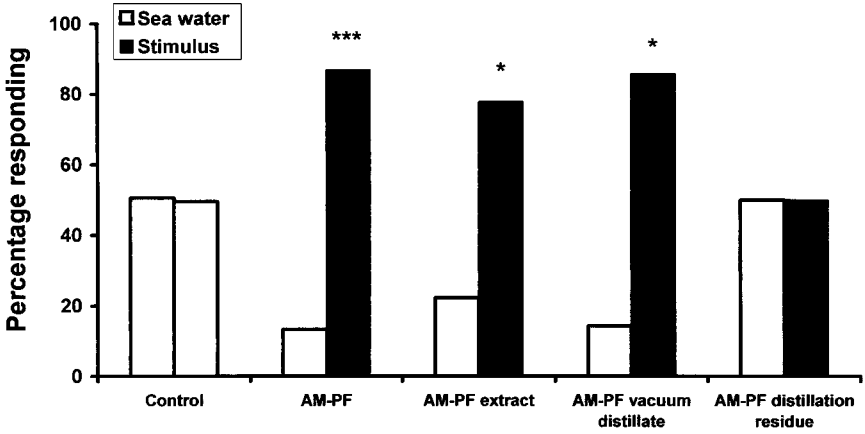


FIG. 2. Taxis response of adult male (AM) sea lice to preadult II virgin female (PF) -derived stimuli in a Y-tube arena. Seawater control, $N = 93$; PF conditioned water, $N = 68$; PF extract, $N = 18$; PF vacuum distillate, $N = 14$; PF distillation residue, $N = 4$; $^{*}P < 0.05$; $^{***}P < 0.001$ (χ^2 analysis). χ^2 analysis was not performed on the results for the PF distillation residue, as numbers were low due to very low activation to the stimulus (see Table 1).

responses to either of these (Table 1, Figure 3). In a competitive assay, in which males were presented with PF or AF in separate arms of the Y tube, there was a significant response to PF ($P < 0.05$) (Figure 3). Although the PF did not show an overall significant increase in activity in response to AM (Table 1), there was an attraction to the males with all 11 of the PF that showed "high activity" levels choosing the treatment arm ($P < 0.01$) (Figure 3).

DISCUSSION

Chemical communication is important in the mating process of many copepods. It is believed that contact chemoreception aids the males of copepods and harpacticoids in distinguishing female developmental stage (Ritchie et al., 1996; Hull et al., 1998; Kelly et al., 1998). Sea lice, *L. salmonis*, use chemoreception to detect a potential host (Devine et al., 2000; Ingvarsdóttir et al., 2002). In this study, they have been shown to be capable of detecting potential mates by the same means. From these findings, it can be concluded that adult *L. salmonis* use both distant and close-range chemical cues to locate a mate. Experimental work on the mate-finding behavior in the Copepoda has mainly concentrated on 3D tracking of individuals (adult males and unmated females) and observations on mate guarding (Katona, 1973; Griffiths and Frost, 1976; Lonsdale et al., 1988, 1998; Maier, 1995; Anstensrud, 1992; Strickler. 1998; Tsuda and Miller, 1998; Doall et al., 1998; Frey

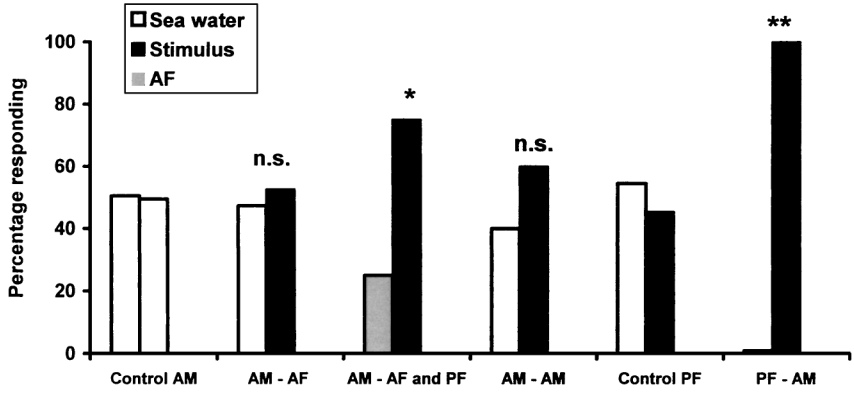


FIG. 3. Taxis response of adult male sea lice (AM) to adult mated females (AF), AF in one arm and preadult II females (PF) in the other, AM, and responses of PF to AM. Seawater control, $N = 93$ AM, $N = 11$ PF; AF conditioned water, $N = 19$; AF and PF conditioned water, $N = 20$; AM conditioned water for males, $N = 20$; AM conditioned water for PF, $N = 11$, $^{*}P < 0.05$; $^{**}P < 0.01$ (χ^2 analysis).

et al., 1998; Hull et al., 1998; Kelly et al., 1998; Lonsdale et al., 1998; Weissburg et al., 1998). In this study, activation of individuals and directional responses of sea lice to other individuals were recorded. Upstream positive rheotaxis of adult male lice to preadult II virgin females was established. Several different species of Copepoda have been found to follow host- and mate-related semiochemical trails, and species-specific mate recognition systems have been found for several species (Blades and Youngbluth, 1980; Frey et al., 1998; Lonsdale et al., 1998; Devine et al., 2000; Ingvarsdóttir et al., 2002 and unpublished). To further support the trail following of the different species of Copepoda, Yen et al. (1998) showed that the low Reynolds number regime of zooplankton conserves distinct species-specific cues affecting their behavior, and where viscosity limits forces to the molecular scale, trails of diffusible odor persist. Further, Dusenbery and Snell (1995) suggested that efficient use of diffusible pheromones was related to the size of the organism and showed that copepods are above the minimum size for diffusible pheromones that markedly improve mate location probabilities.

The presence of females alters male behavior of the harpacticoid *Tigriopus japonicus* (Kelly et al., 1998). The adult males showed a preference only for conspecific virgin females as partners. This supports the findings of Hull (1997), Ritchie et al. (1996) and Hull et al. (1998) on mate guarding in *L. salmonis*. In our study, male sea lice were activated by the presence of different developmental stages, preadult II virgin females, adult mated females, and other males. However, males of *L. salmonis* could distinguish between adult mated female stimuli and preadult II virgin female stimuli, with over 75% of the males choosing the virgin females. The males were also directionally attracted to preadult II virgin female

SPE extract and the vacuum distillate of the extract but not to adult mated females or to adult males. The vacuum distillate comprised components with a molecular weight range comparable to those of compounds utilized as volatile semiochemicals by terrestrial organisms, while the residue arising from the vacuum distillation comprised components with little or no volatility. Attraction of the males to only the vacuum distillate supports our initial hypothesis that sea lice would use low-molecular-weight diffusible pheromones for mate location and is also in agreement with other postulations on the molecular size of the components of copepod sex pheromones (Lonsdale et al., 1998). Production of a diffusible pheromone by virgin females may cease immediately upon mating (Lonsdale et al., 1998; Kelly et al., 1998), which also is in agreement with our study, as males were not directionally attracted to adult mated females. It is, therefore, not contact pheromones that attract the males initially, but long distance chemical cues that initiate their search for virgin females.

Adult male lice were activated by odors from other adult males and adult mated females but did not show upstream rheotaxis behavior to these chemical cues. A proportion of the preadult II virgin females showed such behavior to adult males, but there was no obvious activation response from the females as a whole. The significance of these findings is not yet understood as they are too preliminary to draw any conclusions. However, it should not be ruled out that other pheromonal components signaling the presence of conspecifics or potential mates may also be present in *L. salmonis*.

In order to incorporate these findings into integrated pest management strategies, it is important to gather further information on the precise chemical composition of the diffusible virgin female-produced pheromones and to test individual compounds to determine the most attractive elements within the preadult II virgin female extract. This would clarify the role of the chemical cues involved in mate location in *L. salmonis.* Such work is currently being carried out.

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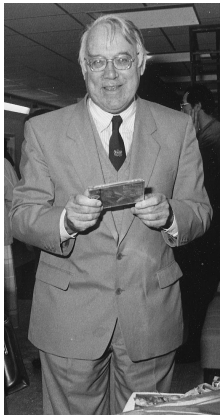
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OBITUARY

JEFFREY B. HARBORNE (1928–2002)



Jeffrey Harborne, who died July 21, 2002, was irrevocably linked to phytochemistry. He was associated with the journal *Phytochemistry* for 32 years, serving both as executive editor, and as editor-in-chief from 1986-1999. He was founder of the journal *Phytochemical Analysis*, and series editor of *Methods in Plant Biochemistry*. His influence on the field of chemical ecology is both seminal and enormous. Many readers of this journal may have first encountered his work in his book *Introduction to Ecological Biochemistry*, now in its 4th edition, and used worldwide as a text to introduce students to chemical ecology and as a reference by scientists working in natural products chemistry and ecology alike. This book probably has done more to foster interest in chemical ecology than any other single publication in the field. It has been the inspiration for countless studies in our field. He was a pioneer and a giant.

Educated at Wycliffe College and the University of Bristol, he developed a life-long interest in flavonoids. He later pursued research on plant pigments at UCLA where he worked with T. Geissman. For many years he was a biochemist at the John Innes Institute, Hertford, UK during which time, with Tony Swain,

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he was instrumental in the establishment of what later became the Phytochemical Society of Europe. He formed a long-term collaboration with Vernon Heywood, and since 1968 he was at the Botany Department at the University of Reading. He authored over 270 research papers and reviews, and was author, editor, or co-editor of 40 books (including *Phytochemical Methods*, *Plant Chemosystematics* with B.L. Turner, *A Dictionary of Plant Toxins*, and 5 others on flavonoids. He was a frequent contributor to this Journal.

Professor Harborne's many honors included being the recipient of: the Silver Medal for life time achievement of the International Society of Chemical Ecology, the first Silver Medal from the Phytochemical Society of Europe, the Gold Medal in Botany from the Linnean Society of London, the first joint award of the Phytochemical Societies of Europe and America honoring lifelong contributions, the Pergamon Phytochemistry Prize for plant biochemistry, and election to Fellowship of the Royal Society. He was an invited speaker at countless international symposia, and he spent time as a visiting professor in Brazil, Texas, California, and Illinois.

Whether or not you were privileged to know Jeffrey Harborne, you are nevertheless indebted to him. His admirers and many friends among the readers of the Journal and the membership of the ISCE will certainly be recalling some personal anecdote that is a bittersweet reminder of what we will miss. His passing touches us, not only because of our wonder at his myriad scientific accomplishments, but also because he was an exceptional man. He was gentle and generous and had a humble and self-effacing nature that is rare. He was also a devoted Anglican who worked for many years as warden of Christ Church in Reading.

We dedicate this issue to his memory.
John T. Romeo, Editor

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Arabidopsis ECOTYPE VARIABILITY IN CAMALEXIN PRODUCTION AND REACTION TO INFECTION BY *Alternaria brassicicola*

I. A. KAGAN* and R. HAMMERSCHMIDT

Department of Plant Pathology
Michigan State University
East Lansing, Michigan 48824-1312, USA

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Abstract—Camalexin production was compared in 24 ecotypes of *Arabidopsis thaliana*. Detached *Arabidopsis* leaves were inoculated with *Cochliobolus carbonum*, an incompatible pathogen of *Arabidopsis*, to test the ability of each ecotype to produce camalexin. Whole plants were inoculated with *Alternaria brassicicola*, a crucifer pathogen, to determine if there was a correlation between the ability of an ecotype to produce camalexin and its resistance to *A. brassicicola*. All ecotypes were capable of producing camalexin, but the amounts produced relative to the Columbia ecotype (used as a standard) varied within and among ecotypes, and among experiments. Different degrees of resistance to *A. brassicicola* were observed among ecotypes, both macroscopically and microscopically. Extraction of *A. brassicicola*-inoculated leaves revealed that only four ecotypes (two resistant and two susceptible) produced easily detectable amounts of camalexin in response to this pathogen. TLC plate bioassays suggested that *A. brassicicola* was relatively insensitive to camalexin, thus casting some doubt on the importance of this compound in defense. These studies suggest that the role of camalexin in disease resistance varies among different *Arabidopsis* populations in nature, and they provide some clues to other possible determinants of resistance to *A. brassicicola*.

Key Words—*Arabidopsis thaliana*, ecotypes, *Cochliobolus carbonum*, *Alternaria brassicicola*, camalexin, disease resistance, microscopy, thin-layer chromatography, bioassays.

* To whom correspondence should be addressed. Current address: USDA-ARS-NPURU P.O. Box 8048 Oxford, MS 38677. E-mail: ikagan@olemiss.edu

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INTRODUCTION

Phytoalexins are low-molecular-weight antimicrobial compounds that accumulate in plants after infection by microorganisms (Paxton, 1981). Since the postulation of their existence by Müller and Börger in 1940, and the first isolation of a phytoalexin in 1960 (reviewed in Deverall, 1982), over 350 phytoalexins have been found in about 30 different plant families (Kuć, 1995; Hammerschmidt, 1999).

Phytoalexins within a plant family are often in the same chemical class (Hammerschmidt, 1999). Those of the family Brassicaceae (Cruciferae), for example, consist of an indole ring with a sulfur-containing moiety (Pedras et al., 1997). The cruciferous weed *Arabidopsis thaliana* produces camalexin (Figure 1) as its principal phytoalexin (Tsuji et al., 1992). This is also the principal phytoalexin of the crucifers *Camelina sativa* (Browne et al., 1991), *Arabis lyrata* (Zook et al., 1998), and *Capsella bursa-pastoris* (Jimenez et al., 1997). As with other phytoalexins, the antimicrobial nature (Browne et al., 1991; Ayer et al., 1992; Tsuji et al., 1992; Conn et al., 1994; Rogers et al., 1996; Pedras and Khan, 1997; Pedras et al., 1998) and *de novo* synthesis of camalexin upon infection (Tsuji et al., 1992; Glazebrook and Ausubel, 1994; Callaway et al., 1996; Zhao and Last, 1996; Jimenez et al., 1997; Mhiri et al., 1997; Zook et al., 1998; Sigareva and Earle, 1999; Thomma et al., 2001) suggest that it may play a role in disease resistance.

The initial approach to determine the role of camalexin in defense against pathogens was to isolate camalexin-deficient mutants and compare their disease phenotypes to that of the wild-type. Five such phytoalexin-deficient *(pad)* mutants of *Arabidopsis*, each representing a mutation in a different gene, have been isolated from ethyl methane sulfonate (EMS) -mutagenized seed of the Columbia-0 and Columbia-glabrous ecotypes (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997).

To date, none of the *PAD* genes is known to be specific to camalexin biosynthesis, although *PAD3* is a strong candidate (Zhou et al., 1999). The *PAD4* gene, which has been isolated (Jirage et al., 1999), is a regulatory gene that stimulates salicylic acid (SA) production in response to certain pathogens (Glazebrook et al., 1997; Zhou et al., 1998). SA, in some, but not all cases (Nawrath and Métraux, 1999), potentiates camalexin production (Zhao and Last, 1996; Zhou et al., 1998).

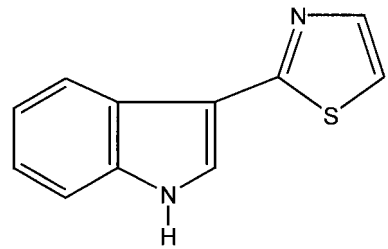


FIG. 1. Camalexin, 3-(2-thiazolyl)indole.

Since SA is required for many defense responses (Vernooij et al., 1994), the disease phenotype of *pad4* could reflect the absence of those other responses and not the absence of camalexin. Because EMS can cause mutations in many different genes (see Robbelen, 1966; Koornneef and van Loenen Martinet, 1983; for examples), some of the other *pad* mutants could also be affected in genes not directly related to camalexin biosynthesis.

As an alternative approach to determining the contribution of camalexin to disease resistance, we examined natural variation in *Arabidopsis* populations. Many ecotypes of *A. thaliana* are available, and although similar enough to be considered one species, they differ genetically in some respects because of selection pressures imposed by the different environments of origin. These differences can include variation in resistance to certain pathogens. For example, different ecotypes of *Arabidopsis* differed in their ability to resist infection by the slime mold *Plasmodiophora brassicae* (Fuchs and Sacristan, 1996), cauliflower mosaic virus (Leisner and Howell, 1992; Callaway et al., 1996), turnip crinkle virus (Dempsey, 1996; Dempsey et al., 1997), the bacterium *Xanthomonas campestris* pv. *campestris* (Tsuji et al., 1991), and the oomycetes *Albugo candida* (Holub et al., 1995) and *Peronospora parasitica* (Mauch-Mani et al., 1993). In those cases, resistance was due to the presence of one or more resistance (R) genes. The types of R genes varied among ecotypes, which may have been a reflection of the disease pressure on the location where each ecotype was collected.

Plant species in different environments also differ in the types of compounds that they produce. For example, the same pine species, in different locations, will qualitatively and quantitatively produce different piperidine alkaloids (Gerson and Kelsey, 1998). Plant species within a genus may also vary in one type of secondary metabolite. Some oat species produce avenacins, which are saponins that provide resistance to the wheat pathogen *Gaeumannomyces graminis* var. *tritici*. Other oat species are avenacin-deficient and are susceptible to that pathogen (Osbourn et al., 1994). Such location- and species-dependent variability in secondary metabolite production may reflect differences in selection pressures of the habitats from which the species originated.

Similarly, the absence of camalexin production in some *Arabidopsis* ecotypes may reflect differences in selection pressures of their original habitats. Since camalexin is not effective against all pathogens (Rogers et al., 1996; Pedras et al., 1997), it is reasonable to think that genes for camalexin production would be lost in habitats where it was useless. However, as genes for production of precursors might remain if any conferred a selective advantage in a habitat, a survey of ecotypes could yield clues about the biosynthesis as well as the physiological role of camalexin.

In this study, we looked for variation in camalexin production among *Arabidopsis* ecotypes and for possible correlations with differences in response to a crucifer pathogen. Twenty-four ecotypes of *A. thaliana* were screened for the ability to produce camalexin when inoculated with the fungus *Cochliobolus carbonum*,

a maize pathogen that induces high concentrations of camalexin in the Columbia ecotype of *Arabidopsis* (Glazebrook et al., 1997; Zook and Hammerschmidt, 1997). At the same time, symptoms that developed after inoculation with the fungus *Alternaria brassicicola*, a common crucifer pathogen (Ellis, 1968), were evaluated. Correlations were sought between resistance to *A. brassicicola* and the ability to produce camalexin.

METHODS AND MATERIALS

Reagents and Chemicals. Camalexin was prepared from a mixture of *Arabidopsis* extracts and from two chemical syntheses. Both syntheses followed the method of Ayer et al. (1992), except that in one case, tetrahydrofuran was used as a solvent instead of benzene. The pooled camalexin was purified by preparative TLC (Whatman plates, 1000 μm thickness) and recrystallized from hexanes–acetone (4:1, v/v) to give a final yield of 20 mg. The purity and concentration of a sample were checked by HPLC by using the method of Glazebrook et al. (1997). Part of the purified camalexin was redissolved in methanol to give a 0.5 mg/ml solution, which was stored at -20°C. Indole-3-carboxaldehyde was purchased from Sigma or Aldrich Chemical Companies and used without further purification (Sigma) or after recrystallization from 95% ethanol (Aldrich). Indole-3-carboxylic acid was obtained from Aldrich. Solvents used for HPLC were of HPLC grade, as was the methanol used for TLC. All other chemicals were of reagent grade or better.

Plant Material and Plant Growth Conditions. The following *Arabidopsis* ecotypes were purchased from Lehle Seed Company (Round Rock,Texas, USA): Aua/Rhön, Bensheim, C24, Cape Verde, Columbia-0, Columbia-glabrous, Dijon G, Estland, Greenville, Kendalville, Landsberg erecta, Mühlen, Niederzenz, Nossen, RLD, RLD1, S96, Turk Lake, and Wassilewskija. Ecotypes Ksk-1, Kas-1, Wei-1, and WA-1 were provided by S. Somerville (Carnegie Institute, Washington, DC, USA). Ecotype Anna was provided by J. Conner (Kellogg Biological Station, Michigan State University, Hickory Corners, Michigan, USA). Seeds were stored over desiccant at room temperature or at 4°C.

Plants were grown in clay pots of 10 or 14.5 cm diameter. Soil (Baccto High Porosity Professional Planting Mix) was wetted with Peters Professional Fertilizer (2.49 g/liter) and covered with a 1- to 2-mm layer of fine vermiculite. Three days after germination, seedlings were fertilized with about 20 ml of fertilizer. Fertilization was repeated once a week, with the amount increasing to about 150 ml/pot by the third week. Plants were grown at 21°C on a 16 hr light regime.

Fungal Cultures and Inoculations. Cochliobolus carbonum (ATCC 90305) and *Alternaria brassicicola* (Trail and Koller, 1993) were grown on V-8 agar according to Tuite (1969, recipe 248) with the following modifications: 160 ml V-8 juice, 1 g calcium carbonate, and 14 g agar were used. Seven- to 14-day-old cultures, grown at room temperature on a 24-hr light regime, were used. For inoculations with *C. carbonum*, excised leaves were inoculated on the abaxial side with

0.1 ml of a spore suspension (2.0×10^5 spores/ml), and incubated in Petri dishes lined with moistened filter paper. *A. brassicicola* spores (2.0×10^5 spores/ml in water + 0.05 % Tween-20) were sprayed onto intact plants with an atomizer. To maintain a humid environment conducive to fungal infection, plants were covered for 24 hr with moist plastic bags, which were then slit to allow gradual equilibration (about 36 hr) with the outside environment. Symptoms were first recorded about 60 hr after inoculation, when bags usually were removed. Symptoms were then recorded daily for 3–7 more days.

Cytological Analysis of A. brassicicola Infection. Leaves of one susceptible and two resistant ecotypes, and of one ecotype with an intermediate degree of resistance, were excised 19, 48, and 72 hr after inoculation. Leaves were placed in glass Petri dishes lined with 2 layers of Whatman No. 1 or No. 4 filter paper saturated with ethanol–acetic acid 3:1 (v/v), which removed pigments and fixed the tissue (Zeyen et al., 1995). Additional solution was pipetted over leaves once or twice a day to keep them from drying. After a few days, ethanol alone was added to the leaves. Prior to microscopy, leaves were rinsed 3–5 times with deionized water (changed every 3–12 hr over 1–2 d), mounted in glycerol on microscope slides, and stored at 4°C. Light micrographs were taken with a Leica microscope at 400× magnification, using Nomarski differential interference contrast (DIC) optics.

Extraction of Camalexin from Inoculated Leaves. For each sample of leaves inoculated with *C. carbonum*, inoculum droplets from 10 leaves (total volume of 1 ml) were collected 24 hr after inoculation, and after dilution with one-half volume of water, the leaf diffusates were extracted 3 times with an equal volume of ethyl acetate. Although camalexin is frequently extracted by boiling leaves and droplets together in 80% methanol (Glazebrook et al., 1997), the droplet extraction method was used because it allowed more rapid sample preparation. Since at the inoculum concentration used, the majority of camalexin is in the leaf diffusates (Kagan and Hammerschmidt, 2001), the camalexin in droplets was considered an accurate representation of the amount in the entire leaf. The pooled organic phases were evaporated to dryness under reduced pressure at 40°C, redissolved in 1 ml methanol, transferred to glass tubes, evaporated under a stream of nitrogen at 40–60°C, and stored at 4°C or −20°C.

To examine camalexin production in response to *A. brassicicola*, plants were sprayed with a spore suspension containing 2×10^5 spores/ml. At 48 hr after inoculation (a time when camalexin concentrations were usually high in *C. carbonum*-inoculated plants), 0.1–0.7 g of leaves (6–15 leaves per replicate in one experiment, and 50–75 leaves per replicate in another) were excised for extraction. The leaves were weighed and boiled in 80% methanol (15 ml/0.1 g tissue) for 20 min. Samples were stored at 4°C for 24–60 hr and then concentrated under reduced pressure at 40–45°C to remove the majority of the methanol. The concentrated extract was diluted with one-half volume of water and then extracted 3 times with an equal volume of chloroform. The pooled chloroform fractions were dried over anhydrous sodium sulfate and then evaporated and stored as described for the leaf diffusate extracts.

Thin-Layer Chromatography (TLC) of Extracts. Droplet extracts were redissolved in 35 μl of ethyl acetate, and the entire sample was loaded (in 5-μl aliquots) onto washed and activated silica gel G TLC plates [20 $\times$ 20 cm, silica thickness: 200 μm on plastic-backed plates (Selecto Scientific), and 250 μm on glass-backed plates (Analtech)]. Leaf extracts were dissolved in 35–60 μl of methanol, depending on the starting amount of leaf tissue. Standards of camalexin (5 μg), as well as the putative precursors indole-3-carboxaldehyde and indole-3-carboxylic acid (usually 1.25–5 μg, and 2.5–5 μg, respectively) were also loaded. Plates were developed once in chloroform and then twice in chloroform–methanol 19:1 (v/v).

The TLC plates were photographed with Kodak slide film (100 ASA) under long-wave or short-wave ultraviolet light, with an Olympus OM-1 camera. A Kodak Wratten filter for blue light covered the camera lens. Photographing plates required a 1- to 1.5-min exposure under long-wave UV, and a 2.5-min exposure under short-wave UV light.

Bioassays for Evaluation of Camalexin Production in Response to C. carbonum and A. brassicicola. To provide a visual record of camalexin production among the different ecotypes, as well as of other antimicrobial compounds, TLC plates were sprayed with a suspension of *Cladosporium cucumerinum* spores (usually 3–3.5 $\times$ 10^6 spores/ml) in half-strength potato-dextrose broth (per liter: 100 g potatoes, 5 g dextrose) and incubated in a humid chamber for 3 d. Fungal hyphae and spores are produced everywhere on the plates except in areas containing antifungal compounds. TLC plates containing extracts of *A. brassicicola*-inoculated leaves were sprayed with *A. brassicicola* (5×10^5 to 1×10^6 spores/ml). Cultures used for bioassays were 7–11 days old.

Quantification of Camalexin. Camalexin was quantified from the bioassayed plates by assuming a directly proportional relationship between the radius of a zone of inhibition (ZOI) and the logarithm of the mass of compound present (Lazarovits et al., 1982). The ZOIs at the R_f of camalexin were traced onto paper and scanned with a Hewlett-Packard ScanJet 4c scanner (resolution of 150 dots per inch in the horizontal and vertical directions). The area in pixels was determined with the Jandel Sigma-Scan program. These numbers were converted into square centimeters based on the number of pixels in standards of known size. The radius (r) of the zone of inhibition was calculated from the formula $A = \pi r^2$, and the mass of camalexin was determined from a standard curve (Figure 2) of inhibitory zone radius versus $\log_{10}$ of mass.

RESULTS

Phenotypic Characterization of Responses to Alternaria brassicicola. The traits used to indicate disease resistance varied between ecotypes and experiments. An ecotype could appear susceptible because of extreme chlorosis on one occasion

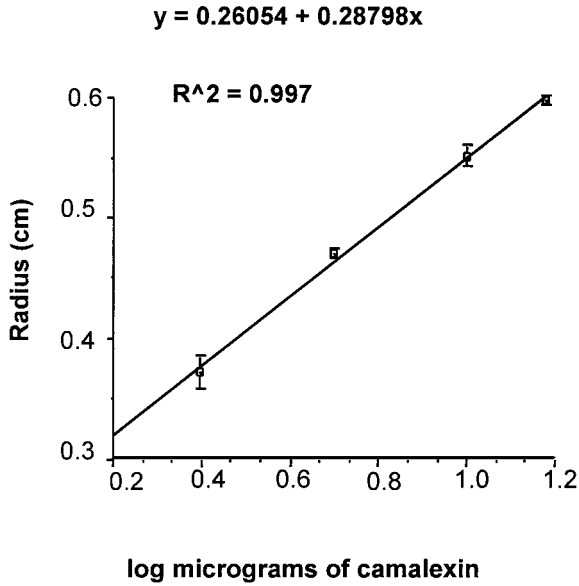


FIG. 2. Relationship between $\log_{10}$ of micrograms of camalexin and radius of zone of inhibition on TLC plates bioassayed with *Cladosporium cucumerinum*. Mean radii were determined from zones of inhibition on two separate TLC plates, onto which known amounts of camalexin were loaded.

and because many large lesions formed on another occasion. Consequently, the ecotypes in Table 1 were grouped by degree of resistance (high, intermediate, or low), and not by a range of disease scores. Phenotypic traits seen consistently in an ecotype were noted.

In general, symptoms of *A. brassicicola* infection consisted of circular brown or gray lesions (1–20 per leaf), 0.5–1.5 mm in diameter. The larger lesions were sometimes irregularly shaped and possibly consisted of several smaller lesions that had coalesced. Chlorosis, although sometimes induced by mock inoculation, was associated primarily with infection. Three general types of chlorosis were observed: (1) yellowing of the entire lesion-containing area with no clear point of origin, (2) yellowing that radiated outward from the leaf midrib to cover the lesion-containing area, or (3) a yellow halo around the lesion. The latter is fairly typical of *A. brassicicola* infection (Ellis, 1968; Conn et al., 1988).

On some plants, infected leaves wilted and became necrotic 3-7 days post-inoculation (dpi). Even on plants with no apparent symptoms of infection, it was common to find at least one of the oldest leaves dead 3–5 dpi. Older leaves seemed more susceptible than younger leaves to *A. brassicicola*.

TABLE 1. ECOTYPES STUDIED FOR CAMALEXIN PRODUCTION AND *A. brassicicola* RESISTANCE: NAME AND/OR ABBREVIATION, ORIGIN, AND DISEASE PHENOTYPE

Ecotype name	Origin	Degree of resistance[a] and distinctive phenotypic traits, if any
Anna	USA	High resistance, symptoms very rare
Bensheim (Be)	Germany	High
Greenville (Gre)	USA	High
Kendalville	USA	High
Ksk-1	Keswick, England	High
Muehlen (Mh)	Poland	High
Nossen	Germany	High
RLD	Rschew, Russia	High; leaves sometimes developed lesions but seldom became chlorotic
S96	Koorneef Group, Netherlands	High
Turk Lake	USA	High; symptoms rare
Aua/Rhön (Aa)	Germany	Intermediate resistance
Cape Verde (Cvi)	Cape Verde Islands	Intermediate; large lesions and profuse chlorosis
Columbia-0 (Col-0; no genetic markers)	USA	Intermediate; variable symptoms (<5% to 15% diseased leaves)
Estland (Est)	Estland, former USSR	Intermediate
Landsberg erecta (La-er)	Germany	Intermediate
Niederzenz (Nie)	Germany	Intermediate to low; lesions usually small (<0.5–1 mm diam.) and numerous (5–10 per diseased leaf)
RLD-1	Rschew, Russia	Intermediate to high; large necrotic zones following lesion development
WA-1	Warsaw, Poland	Intermediate; large circular lesions
Wassilewskija (Ws)	Russia	Intermediate
Wei-1	Weiningern, Switzerland	Intermediate to low; round lesions about 1.5 mm diam.
C24 (glabrous strain used in transformation)	Collected by B. Damm	Low resistance; large lesions, usually at margins
Columbia-glabrous (Col-gl; glabrous leaves used as a genetic marker)	USA	Low; large lesions at margins and rapid development of wilting and necrosis
Dijon G (Di-G)	Dijon, France	Low
Kas-1	Kashmir Province, India	Low

[a] High resistance: lesions on fewer than 10% of leaves; little chlorosis. Intermediate resistance: lesions and necrotic/chlorotic areas on 10–40% of leaves. Low resistance: large lesions and profuse chlorosis; symptoms affecting 50-100% of leaves by end of observation period.

Highly resistant plants had lesions on fewer than 10% of the leaves, and infected leaves were seldom chlorotic. Dead rosette leaves were sometimes the only indications of pathogen infection. Plants with intermediate resistance had lesions and necrotic/chlorotic areas that could be quite large, but on only 10–40% of the leaves. This category included plants with gradually increasing numbers of diseased leaves during the observation period. Plants with low resistance had large lesions on many leaves, frequently accompanied by profuse chlorosis, at the start of the observation period. Up to 50–100% of the leaves were infected by the end. Necrosis, wilting, and death in the most susceptible plants were observed by 3–4 dpi.

The degree of resistance or susceptibility in each ecotype depended partly on light regime and inoculum concentration. When plants were grown under less light, some of the normally resistant ecotypes, such as Bensheim, had severe disease symptoms. At a high inoculum concentration (3.5×10^5 spores/ml), almost all of the ecotypes tested (Mühlen, RLD1, RLD, Columbia-0, Greenville, Kendalville, Estland, S96, Aua/Rhön, Nossen, and Anna) with the exception of Anna, appeared susceptible. At a lower inoculum concentration (1.0×10^5 spores/ml), all plants tested (Anna, Columbia-0, Columbia-glabrous, Cape Verde, C24, Ksk-1, Landsberg erecta, Niederzenz, Wei-1, Wa-1, Wassilewskija, and Kas-1) appeared resistant except for Niederzenz, Wei-1, and Wassilewskija.

Symptoms could vary considerably between experiments. For example, although Dijon G consistently had lesions and chlorotic leaves, it sometimes had large lesions within chlorotic areas or had many small necrotic lesions less than 0.5 mm in diameter.

Cytological Analysis of Infection in Resistant and Susceptible Ecotypes. No striking differences were seen in numbers of conidia per square centimeter on the surfaces of susceptible and resistant ecotypes, although some clusters of conidia created localized differences. For example, although the micrographs in Figures 3 and 4 show about 6 conidia on the surface of Turk Lake, and 3 conidia on the highly susceptible Kas-1 and highly resistant RLD, the average numbers of conidia per leaf were similar. Therefore, resistance to *A. brassicicola* was not due to discrepancies in the amount of viable inoculum. More differences were noticed in hyphal development and tissue response. On the resistant Turk Lake (Figure 4A), conidia formed unusually short germ tubes, and penetration events were rare. On the resistant RLD (Figure 4B), germ tubes were about as long as on the susceptible Kas-1 (Figure 3). However, fewer lesions and less browning were seen in RLD than in Kas-1 cells. Micrographs of RLD leaves viewed under UV light (not shown) revealed a fluorescent orange material permeating the tissue, suggesting the presence of phenolic compounds.

Camalexin Production in Response to C. carbonum. Because compatible pathogens sometimes do not elicit phytoalexin production (Zhao and Last, 1996),

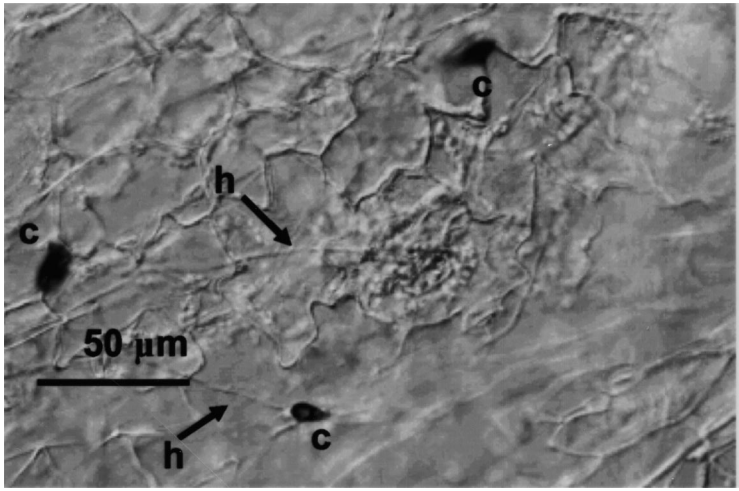


FIG. 3. Light micrographs (400× magnification, Nomarski optics) of ecotype Kas-1 (low degree of resistance) 48 hr after inoculation. One of the 3 conidia (c) has germinated, forming a long hypha (h) extending into a cell.

an incompatible pathogen was needed as a neutral inducer. *C. carbonum* is an incompatible pathogen of the Columbia ecotype and known to elicit large quantities of camalexin (Glazebrook et al., 1997). Quantifying camalexin from the areas of ZOI's on bioassayed TLC plates, although less accurate than the HPLC method used previously (Kagan and Hammerschmidt, 2001), provided a quick visual assessment of ecotype variability in production of camalexin and of other antimicrobial compounds.

As seen in Figure 5, other antimicrobial compounds were detected. However, because they were not seen consistently in different experiments, they were not analyzed further. The putative precursor indole-3-carboxylic acid (approximate $R_f = 0.2$–0.3) was detected under short-wave UV light in most extracts. Whether it was present as a precursor or metabolite was not determined. It was not antimicrobial (Figure 5); nor was the indole-3-carboxaldehyde (Figure 5), another putative precursor that was not detected in the extracts [possibly because indole-3-carboxaldehyde concentrations tend to reach a maximum about 6 hr postinoculation and then decrease (Kagan and Hammerschmidt, data not shown)].

Camalexin production in the different ecotypes (Table 2) was measured relative to Columbia-0 because the latter was used as a wild-type plant in earlier studies (Glazebrook et al., 1997; Kagan et al., 1997). Because the purpose of these experiments was to compare camalexin production among inoculated ecotypes, water-inoculated controls were not included. The variability in the amounts of camalexin found in each ecotype indicated that camalexin was not constitutively

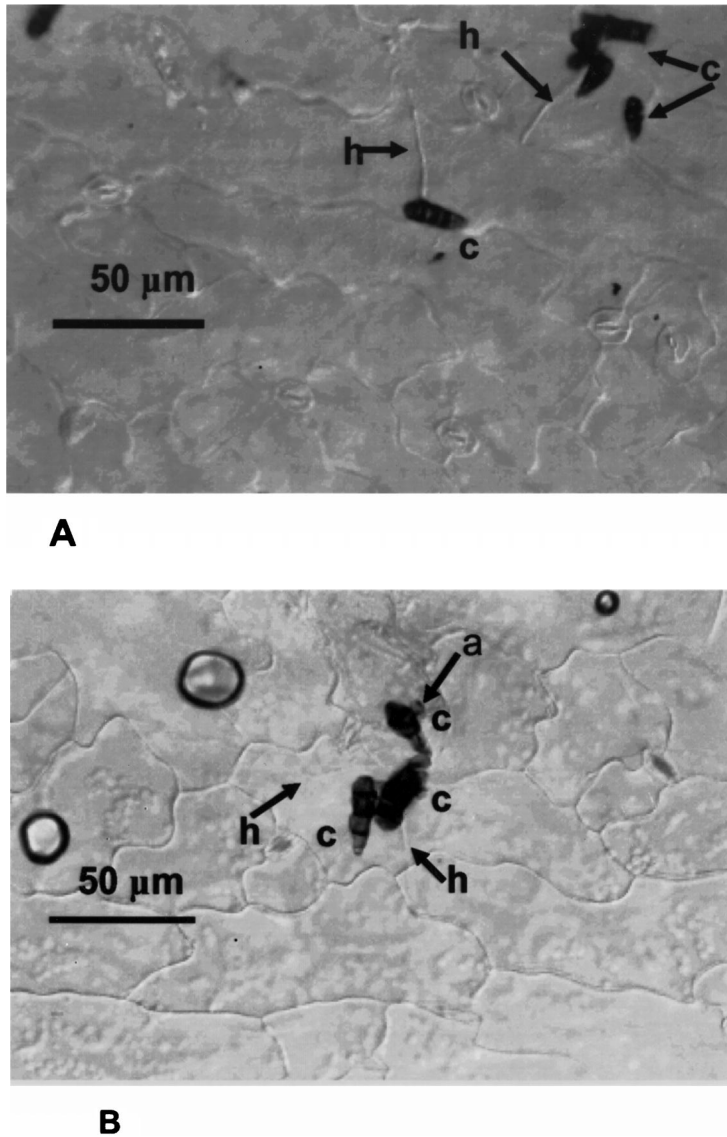


FIG. 4. Light micrographs (400× magnification, Nomarski optics) of ecotypes Turk Lake and RLD (high degree of resistance). (A) Turk Lake, 72 hr after inoculation. Note the short hyphae (h) extending from the conidia (c). (B) RLD, 72 hr after inoculation. The conidium near the top of the figure has formed an appressorium (a) to penetrate the cell directly beneath it. The infected cell appears collapsed. Hyphae are indicated by arrows. Black circles are air bubbles.

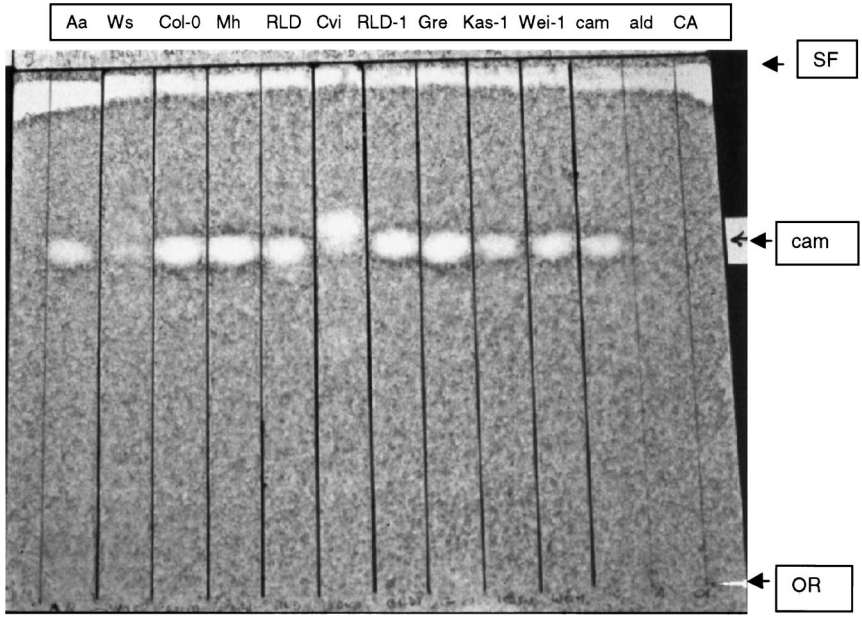


FIG. 5. TLC plate bioassay revealing camalexin accumulation in some *Arabidopsis* ecotypes in response to *Cochliobolus carbonum* (neutral inducer). The plate was sprayed with *Cladosporium cucumerinum* spores and incubated in a humid chamber for 3 d. Ecotypes: Aua/Rhön (Aa), Wassilewskija (Ws), Columbia-0 (Col-0), Mühlen (Mh), RLD, Cape Verde (Cvi), RLD-1, Greenville (Gre), Kas-1, Wei-1. Standards of camalexin (cam), indole-3-carboxaldehyde (ald), and indole-3-carboxylic acid (CA) were also loaded. The location of the camalexin band is shown by the arrow on the right, as are the origin (OR) and solvent front (SF).

produced in any. Eight ecotypes besides Col-0 produced undetectable amounts of camalexin in at least one replicate (Table 2). All ecotypes produced camalexin in at least one experiment (Table 2). However, apart from Dijon G and Wassilewskija, which consistently produced low amounts relative to Col-0 (Table 2, Figure 5), no trends in camalexin production were observed. Some ecotypes were analyzed in 4–5 experiments because they were grown in additional plantings done to obtain data on the poor germinators (Turk Lake, WA-1, and Ksk-1). The extra replicates of ecotypes such as Bensheim, Mühlen, RLD, and RLD-1 confirmed that camalexin production could vary in absolute amounts and in comparison to Col-0. Mühlen, for example, produced 0.73–10 μg in 5 replicates, and the amounts ranged from two-thirds to three times the amount produced by Col-0 in those experiments. Variation in camalexin production relative to Col-0 was due partly to variation in camalexin production in Col-0 itself, which did not always produce detectable amounts

TABLE 2. CAMALEXIN PRODUCTION IN RESPONSE TO *Cochliobolus carbonum*

Ecotype	Range of camalexin produced (μg) per 10 leaves in N replicates[a]	Camalexin concentration, as % of amount produced by Col-0[b]
Anna	0.66–7.2 ($N = 6$)	82–340 ($N = 3$)[c]
Aua/Rhön	0.85–4.2 ($N = 3$)	29–48 ($N = 2$)
Bensheim	0–4.6 ($N = 4$)	380–420 ($N = 2$)
C-24	1.4–3.2 ($N = 4$)	32–210 ($N = 3$)
Cape Verde	0.79–12.5 ($N = 4$)	9.0–230 ($N = 3$)
Columbia-0	0–8.7 ($N = 10$)	not applicable
Columbia-glabrous	0–2.7 ($N = 5$)	12–150 ($N = 4$)
Dijon G	0–1.5 ($N = 5$)	Not determined[d]
Estland	0–2.1 ($N = 4$)	69–120 ($N = 3$)
Greenville	0–8.8 ($N = 4$)	100–200 ($N = 3$)
Kas-1	3.4–5.3 ($N = 3$)	61–310 ($N = 2$)
Kendalville	2.0–3.1 ($N = 3$)	23–35 ($N = 2$)
Ksk-1	1.9–7.1 ($N = 4$)	210–400 ($N = 3$)
Landsberg erecta	1.8–3.3 ($N = 4$)	21–38 ($N = 2$)
Mühlen	0.73–10 ($N = 5$)	67–290
Niederzenz	1.8–4.5 ($N = 4$)	34–120 ($N = 3$)
Nossen	0.40–1.8 ($N = 4$)	18–110 ($N = 3$)
RLD	0–6.6 ($N = 6$)	75–190 ($N = 3$)
RLD-1	2.0–9.0 ($N = 5$)	100–510 ($N = 3$)
S96	2.1–5.6 ($N = 4$)	37–200 ($N = 3$)
Turk Lake	0–6.8 ($N = 4$)	99–130 ($N = 3$)
WA-1	0–2.8 ($N = 3$)	32–140 ($N = 2$)
Wassilewskija	0–1.9 ($N = 2$)	0–22
Wei-1	0.84–5.3 ($N = 4$)	32–190

[a] Leaves were inoculated with *C. carbonum,* and diffusates were extracted after 24 hr. Camalexin on TLC plates was quantified by measuring the area of the zone of inhibition (20I) after bioassay with *Cladosporium cucumerinum*. Mean areas were calculated from 3 scans of a traced ZOI (see Methods and Materials). Replicates were from at least 2 independent experiments. Zeroes indicate an absence of camalexin, or of a measurable ZOI.

[b] Camalexin measured relative to that in the Col-0 ecotype extracted and bioassayed on the same dates.

[c] N values are given in column 3 when different from those in column 2 (occurs if the camalexin relative to Col-0 could not be calculated due to a lack of detectable camalexin in the ecotype or in Col-0).

[d] Quantifiable camalexin was from experiments yielding undetectable amounts in Col-0.

(Table 2). Few correlations were seen between camalexin production in response to a neutral inducer and resistance to *A. brassicicola*. Although the highly susceptible Dijon G was one of the lowest producers, the susceptible ecotypes Kas-1 and C24 produced intermediate amounts of camalexin that were up to 310 % of the amounts produced in Col-0 (Table 2). Furthermore, the highly resistant Nossen produced relatively low amounts of camalexin in absolute (0.4–1.8 μg) and relative terms (18–110 % of Col-0).

Camalexin Production in Response to A. brassicicola. In one experiment, small amounts of camalexin were present in 10 ecotypes (Kas-1, WA-1, Turk Lake, Dijon G, Niederzenz, Anna, RLD, RLD-1, Wassilewskija, Col-0), but in only one of the two replicates. The camalexin band was slightly brighter in Kas-1 and RLD than in the other ecotypes. No zones of inhibition were formed in TLC plate bioassays. When the experiment was repeated with the same ecotypes, minus the Wassilewskija ecotype, and with an approximately fivefold increase in leaf mass, only Kas-1, Anna, and RLD produced enough camalexin to detect under UV light. After bioassay with *A. brassicicola* spores, faint ZOI's at the R_f of camalexin were detectable in extracts from the resistant ecotypes RLD and Anna (which contained two other faint ZOI's, indicating the presence of additional antimicrobial compounds), and from the susceptible ecotypes Kas-1 and Dijon G. The faint ZOI's at the R_f of camalexin may indicate insensitivity of *A. brassicicola* to camalexin, since the ZOI formed by the standard was fainter than on a *C. cucumerinum*-sprayed plate assayed on the same day (not shown).

DISCUSSION

To better understand the role of camalexin in disease resistance, we compared camalexin production in 24 *Arabidopsis* ecotypes and looked for correlations with resistance to *Alternaria brassicicola*, a fungal pathogen of crucifers. Ecotype resistance was classified as high, intermediate, or low (Table 1). These categories encompassed a range of symptoms, which frequently varied within ecotypes from one experiment to the next. This variation in symptoms, within and among ecotypes, suggests that resistance of *Arabidopsis* to *A. brassicicola* may involve multiple defense genes, as found in studies of *Arabidopsis* resistance to *Peronospora parasitica* (Mauch-Mani et al., 1993).

All ecotypes could produce camalexin in response to the incompatible fungal maize pathogen *Cochliobolus carbonum* (Table 2), demonstrating that susceptibility to *A. brassicicola* was not due to an inability to produce camalexin. However, with the exception of the low-producing Dijon G and Wassilewskija, no ecotypes produced consistently high or low amounts of camalexin. The amounts produced in response to this neutral inducer did not, in general, seem correlated with resistance to the crucifer pathogen.

Following *A. brassicicola* inoculation, the only ecotypes producing enough camalexin to inhibit *A. brassicicola* in bioassays were the highly susceptible Kas-1 and highly resistant RLD, and (in one experiment) the highly susceptible Dijon G and highly resistant Anna as well. These results suggest that among the ecotypes tested, camalexin may not be a key factor in resistance to *A. brassicicola*.

Recently, Thomma et al. (1999) demonstrated a role for camalexin in resistance to *A. brassicicola*, based on work with the *pad3* mutant of *Arabidopsis*, which

produces no camalexin in response to this pathogen or to others previously tested (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997). This mutant was more susceptible to *A. brassicicola* than either *pad1* or the wild-type Col-0 ecotype, both of which produced significant amounts of camalexin following *A. brassicicola* inoculation (Thomma et al., 1999). We found that Col-0 produced faintly detectable camalexin in one replicate of one experiment and that the amounts produced did not inhibit *A. brassicicola* in a bioassay. Our results for camalexin production cannot be compared directly to those of Thomma et al., due to differences in bioassay and quantification methods. However, the fact that in two experiments camalexin was detected in both the highly resistant RLD and highly susceptible Kas-1 suggests that for some ecotypes of *Arabidopsis*, factors other than camalexin may be more important in determining resistance to *A. brassicicola*. Other studies of cruciferous phytoalexins have also demonstrated that correlations between phytoalexin production and disease resistance break down when a large number of accessions are examined (Rouxel et al., 1991).

The argument against a primary role for camalexin in resistance to *A. brassicicola* is supported by the relative insensitivity of *A. brassicicola* to camalexin. Faint zones of inhibition in our TLC plate bioassays, suggesting that camalexin poorly inhibited *A. brassicicola* growth, are corroborated by the findings of Thomma et al. (1999) that *A. brassicicola* was completely inhibited by 500 μg/ml camalexin. Although more sensitive to camalexin than *Botrytis cinerea*, which was not inhibited by that concentration, *A. brassicicola* required 5 times more camalexin for complete inhibition than did *Neurospora crassa* (Thomma et al., 1999). Since minimum inhibitory concentrations (MIC's) of camalexin for other pathogens are about 50 μg/ml for *Rhizoctonia solani* (Pedras and Khan, 1997) and 80 μg/ml for *Alternaria brassicae* (Jimenez et al., 1997), it appears that *A. brassicicola* is less sensitive to camalexin than some other tested plant pathogens.

The qualitative results of the infection studies with *A. brassicicola*, along with the results of the bioassays from *C. carbonum*-inoculated plants, lay a foundation for further, more quantitative studies to determine other factors that may be involved in resistance to *A. brassicicola* and the relative role of camalexin among those factors. Camalexin may be required for disease resistance in some ecotypes but not others. Contributing factors to resistance may differ among ecotypes, since cytological analysis of infection revealed different resistance responses. The relatively long hyphae on RLD leaves (Figure 4B) suggested that resistance was due to the synthesis of inducible compounds, such as camalexin, in response to cell penetration. In contrast, the short hyphae on Turk Lake leaves (Figure 4A) suggested inhibition of fungal growth at or near the leaf surface, perhaps due to the presence of preformed antimicrobial compounds. Since ecotype Anna, after inoculation with *A. brassicicola*, contained two faint antimicrobial zones besides camalexin in bioassays, it seems possible that other ecotypes could also contain compounds inhibitory to *A. brassicicola*. For example, the orange fluorescent

material observed by fluorescence microscopy in RLD (see Results) suggested the presence of phenolics, often associated with defense responses and possibly too polar to be separated in the TLC solvent used in this study. As no protein extractions were done, the correlation of resistance with defensins (Penninckx et al., 1996; Thomma et al., 1998, 1999) in the various ecotypes is uncertain. Future work should involve concerted analysis of camalexin, phenolics, and defensins to help determine the relative role of each in disease resistance.

Another possible resistance-determining factor is the response to fungal toxins that may be produced during infection (Knogge, 1996). *A. brassicicola* produces a host-specific toxin that may be an important determinant of pathogenicity (Cooke et al., 1997; Otani et al., 1998). A study of 21 members of the family Brassicaceae demonstrated that following application of toxin-containing spore germination fluids (inoculum droplets filtered to remove spores), the severity of necrosis on leaves was correlated with host susceptibility to *A. brassicicola* (Cooke et al., 1997). More recently, the cause of the necrosis was determined to be a host-specific toxin, named AB-toxin (Otani et al., 1998). The variations observed in ecotype resistance to *A. brassicicola* could reflect the presence or absence of genes for AB-toxin detoxification. Further work should include analysis of AB-toxin concentrations in spore inoculum droplets and a search for correlations between *A. brassicicola* resistance and AB-toxin metabolism.

Future work should also involve studies with other pathogens, as camalexin may have a greater role in ecotype resistance to pathogens sensitive to this compound. It would be interesting to find the compatible and incompatible pathogens that are native to the habitats of the various ecotypes and to look for correlations between resistance, susceptibility, and pathogen sensitivity to camalexin.

This study revealed some factors to consider when comparing camalexin production and pathogen resistance among a wide range of ecotypes. As previously mentioned, other compounds besides camalexin may contribute to resistance, and sensitivity to the pathogen or to its toxin(s) may affect the outcome as well. In addition, thorough kinetic studies need to be done before drawing conclusions about whether an ecotype accumulates high or low amounts of camalexin or other compounds. Earlier studies with Col-0 revealed that plants may accumulate camalexin more slowly on some occasions than on others, thus appearing to be camalexin-deficient if examined at only one time point (Kagan and Hammerschmidt, 2001). In this study, camalexin accumulation in response to *C. carbonum* varied within and among ecotypes. By following that accumulation over several days, HPLC quantification may, in the future, establish clearer differences in production by different ecotypes.

Other factors have the potential to bias the evaluation of resistance to a pathogen. The variations in symptoms observed among experiments, besides suggesting the presence of multiple defense genes, demonstrated the need to consider the model of the disease triangle (Agrios, 1997) when evaluating disease

resistance. Even if a plant has the capacity to resist infection, disease may develop if the environmental conditions are right and the pathogen is sufficiently aggressive. In the case of the ecotypes studied here, differences in light regime or inoculum concentration led to aberrant disease phenotypes. Plants grown under reduced light, and hence somewhat stunted, were generally more susceptible to *A. brassicicola* than plants grown under a normal light regime, and this greater susceptibility may have been due to the fact that the stunted plants, lacking sufficient reserves for normal metabolism, also lacked reserves for defense.

Thus far, the role of camalexin in disease resistance has been evaluated by studying the correlation between camalexin production and disease resistance in a small number of wild-type ecotypes or in the phytoalexin-deficient (*pad*) mutants generated by EMS mutagenesis of the Col-0 or Col-gl ecotypes. Such studies, if expanded to wild-type representatives of a larger number of ecotypes, and in relation to a larger number of pathogens, should provide a more comprehensive picture of the role of camalexin in disease resistance. However, it will be important to base conclusions on a large number of experiments in order to have the most accurate picture of the range of symptom variation, to recognize that inoculum concentrations or simple environmental factors may affect results, and to look for other factors contributing to disease resistance in order to have the best possible evaluation of the relative importance of camalexin.

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ANTIFUNGAL LEAF-SURFACE METABOLITES CORRELATE WITH FUNGAL ABUNDANCE IN SAGEBRUSH POPULATIONS

SHARON M. TALLEY,[1] PHYLLIS D. COLEY, and THOMAS A. KURSAR,*

Department of Biology
University of Utah
257 South 1400 East
Salt Lake City, Utah 84112-0840, USA

[1]*Current address: Center for Turbulence Research*
Building 500, Stanford University, Stanford
California 94305-3030, USA

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Abstract—A central component in understanding plant–enemy interactions is to determine whether plant enemies, such as herbivores and pathogens, mediate the evolution of plant secondary metabolites. Using 26 populations of a broadly distributed plant species, sagebrush (*Artemisia tridentata*), we examined whether sagebrush populations in habitats with a greater prevalence of fungi contained antifungal secondary metabolites on leaf surfaces that were more active and diverse than sagebrush populations in habitats less favorable to fungi. Because moisture and temperature play a key role in the epidemiology of most plant–pathogen interactions, we also examined the relationship between the antifungal activity of secondary metabolites and the climate of a site. We evaluated the antifungal activity of sagebrush secondary metabolites against two fungi, a wild *Penicillium* sp. and a laboratory yeast, *Saccharomyces cerevisiae*, using a filter-paper disk assay and bioautography. Comparing the 26 sagebrush populations, we found that fungal abundance was a good predictor of both the activity ($r^2 = 0.36$ for *Saccharomyces*, $r^2 = 0.37$ for *Penicillium*) and number ($r^2 = 0.34$ for *Saccharomyces*) of antifungal secondary metabolites. This suggests that selection imposed by fungal pathogens has led to more effective antifungal secondary metabolites. We found that the antifungal activity of sagebrush secondary metabolites was negatively related to average vapor pressure deficit of the habitat ($r^2 = 0.60$ for *Saccharomyces*, $r^2 = 0.61$ for *Penicillium*). Differences in antifungal activity among populations were not due to the amount of secondary metabolites, but rather to qualitative differences in the composition of antifungal compounds. Although all populations in habitats

* To whom correspondence should be addressed. E-mail: kursar@biology.utah.edu

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with high fungal prevalence had secondary metabolites with high antifungal activity, different suites of compounds were responsible for this activity, suggesting independent outcomes of selection on plants by fungal pathogens. The location of antifungal secondary metabolites on the leaf surface is consistent with their putative defense role, and we found no evidence supporting other functions, such as protection from ultraviolet light or oxidation. That the antifungal activity of sagebrush secondary metabolites was similar for two different fungi provides support for broad antifungal defenses. The incidence and severity of fungal disease in the field (caused by *Puccinia tanaceti*) were similar in moist and dry habitats, possibly reflecting an equilibrium between plant defense and fungal attack, as sites with greater fungal abundance compensated with more effective secondary metabolites. The geographic correlation between fungal abundance and antifungal secondary metabolites of sagebrush, coupled with our other data showing heritable variation in these metabolites, suggests that pathogenic fungi have selected for antifungal secondary metabolites in sagebrush.

Key Words—*Artemisia tridentata,* selective pressures, plant-pathogen interactions, plant defenses, trichomes, sesquiterpene lactones, *Puccinia tanaceti.*

INTRODUCTION

The idea that herbivores and pathogens shape the chemical defenses of their hosts is central to the field of plant–enemy interactions (Ehrlich and Raven, 1964; Edwards, 1992; Rosenthal and Berenbaum, 1992; Rausher, 1992). Yet surprisingly, there is limited evidence that plant enemies exert enough selective pressure on plants to drive the evolution of novel secondary metabolites (Futuyma, 1983; Jermy, 1984; Marquis, 1992; Berenbaum, 1995). In order to demonstrate natural selection in the wild, Endler (1986) argued that it is necessary to show (1) the existence of heritable variation in the trait, and (2) that variation in the trait explains variation in fitness. Furthermore, although it is difficult to identify conclusively the selective agent without manipulative experiments, strong indirect evidence can be obtained by showing that geographic variation in a putative selective agent is correlated with a parallel variation in the defensive trait.

Studies examining the relationship between herbivores and intraspecific variation in plant secondary metabolites have provided evidence for at least some of Endler's criteria. For example, Berenbaum and colleagues have shown that resistance by wild parsnip to the parsnip webworm is chemically based and genetically controlled, although they did not look to see if variation in webworm selective pressure led to different patterns of defense investment (Berenbaum et al., 1986). Mauricio and Rausher (1997) demonstrated that removal of herbivores was followed by a decrease in the amount of glucosinolates and trichome density in subsequent generations of naturalized *Arabidopsis thaliana*. However, for long-lived organisms, it is often not feasible to experimentally manipulate selective

agents or monitor populations for enough generations to demonstrate changes in the frequency of traits. Thus, some studies have used natural variation in the abundance of herbivores within a plant population and then looked for associated patterns of plant defense. Dolinger et al. (1973) demonstrated that populations of *Lupinus* sp. occurring where their specialist herbivore was abundant produced a greater amount and number of alkaloids than populations occurring in areas where the herbivore was rare. Similarly, studies on monoterpenoids in *Satureja douglasii* (Labiatae) and cyanogenic glycosides in *Trifolium repens* and *Lotus corniculatus* (Fabaceae) illustrate that susceptible plant chemotypes occur in sites having fewer herbivores (Jones, 1972, 1988; Rice et al., 1978; Dirzo and Harper, 1982; others in Table 5.1 of Endler, 1986).

Evolution of plant defenses against pathogens is best understood in the case of gene-for-gene interactions (Clay and Kover, 1996; Simms, 1996). These are mediated primarily by plant signaling pathways that recognize the pathogen and induce programmed cell death, an oxidative burst and phytoalexin production (Hammerschmidt, 1999). The majority of fungal pathogens contact leaves as spores or mycelial fragments and infect a leaf by penetrating the leaf epidermis or by entering a stoma (Edwards, 1992). Consequently, the environment at the plant surface plays an important role in fungal adhesion and development (Tucker and Talbot, 2001). For these reasons, plant surface secondary metabolites may mediate plant–pathogen interactions by protecting the leaf from fungal penetration. Nevertheless, the interactions between constitutive metabolites located on the plant surface and pathogens are little studied (Blakeman and Atkinson, 1981; Anderson, 1982; Atkinson and Blakeman, 1982; Hargreaves et al., 1982; Elleman and Entwistle, 1985; Van Etten et al., 1994; Osbourn, 1999; Hakulinen et al., 1999; Sicker et al., 2000).

In this study, we examined the relationship between fungi and antifungal secondary metabolites that are primarily located in glandular trichomes on the leaf surface of sagebrush, *Artemisia tridentata* (Asteraceae). Sagebrush is a long-lived shrub distributed throughout the Western United States (Ferguson, 1964) and is attacked by a number of pathogenic fungi (Farr et al., 1989). The secondary metabolites of sagebrush have been extensively documented (Shafizadeh and Melnikoff, 1970; Kelsey et al., 1973,1982; Bhadane et al., 1975; Brown et al., 1975), although their role in defense against fungal pathogens has not been examined. Sagebrush produces several classes of secondary metabolites, such as coumarins, flavonoids, monoterpenes, and sesquiterpene lactones, that are primarily sequestered in glandular trichomes on the leaf surface (Kelsey and Shafizadeh, 1980; Slone and Kelsey, 1985). The glandular trichomes have cuticular sacs that cover 21–35% of the leaf surface (Kelsey and Shafizadeh, 1980). The secondary metabolites in the glandular trichomes are likely released onto the leaf surface as the cuticular sac matures. In *Artemisia annua*, a cuticular sac forms from secretory cells, accumulates metabolites, and at maturity the sacs rupture discharging their contents to the leaf surface

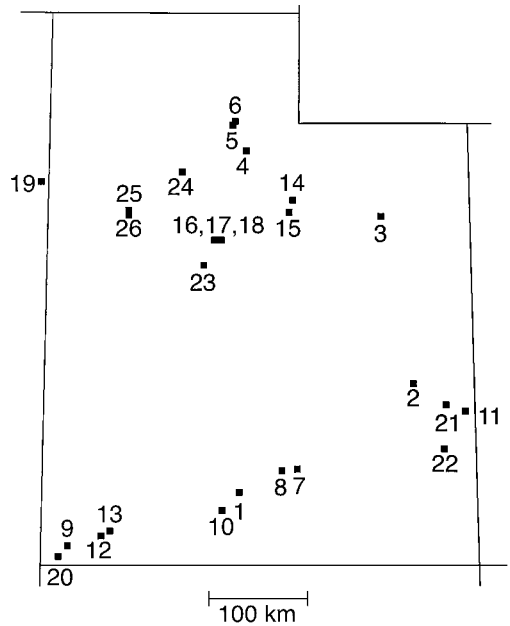


FIG. 1. Geographic locations of the sagebrush populations in and near Utah. Aspect and altitude distinguish sagebrush populations in close proximity.

(Duke and Paul, 1993; Werker, 2000). Sesquiterpene lactones, a major constituent in the glandular trichomes of sagebrush, have been shown to differ among populations (Shafizadeh et al., 1971; Kelsey et al., 1975), but within a given individual, there is no qualitative variation with respect to ontogeny or season (Kelsey, 1974).

Because geographic correlations between a trait and a selective agent are indicative of natural selection, an analysis of geographic variation in host–pathogen interactions is a valuable first step in understanding adaptation (Endler, 1986; Burdon and Thrall, 1999; Lively, 1999; Gomulkiewicz et al., 2000). To test whether selection by fungal pathogens has led to interpopulational differences in the leaf-surface secondary metabolites of sagebrush, we chose 26 populations occurring in diverse habitats and climates in the Intermountain West (Figure 1). Our previous work showed that secondary metabolites of sagebrush had a genetic basis and differed in both number and identity among populations (Talley, 2001). To estimate the potential selective pressure imposed by pathogenic fungi, we measured the fungal abundance and richness within each sagebrush habitat as well as climate conditions likely responsible for fungal prevalence (Talley et al., 2002). We found that fungal abundance and richness varied among populations and were negatively

correlated with vapor pressure deficit (VPD, a measure of the desiccating capacity of the air), temperature, and the average number of consecutive days with maximum diurnal relative humidity $<50\%$, and positively correlated with daily maximum relative humidity (Table 1).

If natural selection has played a role in the evolution of sagebrush antifungal secondary metabolites, among-population variation in these defenses should correspond to the likelihood of attack by fungi. Here, we ask if sagebrush populations in habitats favorable to fungi have a greater activity and a greater number of antifungal secondary metabolites than sagebrush populations in drier habitats. We also examine the possibility that these metabolites might have alternative functions. Finally, we test the prediction that the extent of fungal disease found in the field is a function of both the abundance of fungal pathogens in the sagebrush habitat and the level of antifungal defenses in the sagebrush population. These data, along with our evidence for heritable variation in sagebrush antifungal metabolites (Talley, 2001), will provide a test of the hypothesis that pathogens are important in shaping plant defensive chemistry.

METHODS AND MATERIALS

Study Sites. Twenty-six sites were chosen within the Intermountain West (Figure 1; Appendix A). These included a range of community types: arid grasslands with cacti and yucca; semiarid shrub-steppes with juniper, pinyon pine, and shrubs; moderately arid scrublands with scrub oak; riparian communities with cottonwoods; and subalpine communities with tall forbs, aspen, and spruce.

For 19 of the sites, we recorded the average relative humidity and temperature every 30 min for a complete growing season (mid-March 1999 to mid-October 1999), by using NOMAD dataloggers protected in a weather-proof casing (OM-NOMAD-RH-32; Omega Engineering, Inc; Stamford, Connecticut, USA).

The coolest site (average temperature of 5.6°C for the growing season) had an average VPD of 0.56 kPa. The hottest site (21.8°C) had an average VPD of 2.53 kPa. The average diurnal maximum in relative humidity ranged from 40% to 97% among sagebrush habitats. Higher elevations were generally moister and cooler than lower elevations.

Quantifying Fungal Prevalence. We used the prevalence of fungi in a habitat as an index of the potential selective pressures by fungal pathogens. To quantify prevalence, we measured the abundance and richness of fungi sampled from air and leaf surfaces of nonsagebrush vegetation at each site. We also sampled fungi from the surface of sagebrush leaves to provide another measure of fungal prevalence.

During September of 1998, we measured the fungal abundance and richness on the leaves of sagebrush at 21 of the study sites. Using sterilized scissors,

TABLE 1. RELATIONSHIPS AMONG FUNGAL PREVALENCE, ANTIFUNGAL ACTIVITY OF SECONDARY METABOLITES, AND CLIMATE FOR 26 SAGEBRUSH POPULATIONS

	Relative fungal abundance and richness of the sagebrush habitats		Total antifungal activity of sagebrush secondary metabolites		Antifungal activity per 800 μg of secondary metabolites		Number of antifungal spots from sagebrush bioautography	
	Abundance r^2	Richness r^2	*Penicillium* r^2	*Sacchar.* r^2	*Penicillium* r^2	*Sacchar.* r^2	*Penicillium* r^2	*Sacchar.* r^2
Climate parameters								
Vapor pressure deficit	(−) 0.30[a]	(−) 0.44[b]	(−) 0.61[c]	(−) 0.60[c]	(−) 0.69[c]	(−) 0.67[c]	(−) 0.39[b]	(−) 0.26[a]
Temperature	(−) 0.18	(−) 0.26[a]	(−) 0.42[b]	(−) 0.42[b]	(−) 0.71[c]	(−) 0.70[c]	(−) 0.18	(−) 0.18
Daily maximum relative humidity	(+) 0.43[b]	(+) 0.65[c]	(+) 0.51[c]	(+) 0.40[b]	(+) 0.53[c]	(+) 0.50[c]	(+) 0.41[b]	(+) 0.20
No. of consecutive days with maximum relative humidity < 50%	(−) 0.56[c]	(−) 0.72[c]	(−) 0.42[b]	(−) 0.40[b]	(−) 0.46[b]	(−) 0.44[b]	(−) 0.25[a]	(−) 0.23[a]
Fungal parameters								
Relative fungal abundance	. .	. .	(+) 0.37[b]	(+) 0.36[b]	(+) 0.30[b]	(+) 0.29[b]	(+) 0.12	(+) 0.34[b]
Relative fungal richness	. .	. .	(+) 0.27[a]	(+) 0.27[a]	(+) 0.21[a]	(+) 0.21[a]	(+) 0.11	(+) 0.24[a]

$P < 0.05$, b: $P < 0.01$, c: $P < 0.001$.

[a] Values are coefficients of determination (r^2) with the sign of the slope in parentheses. The relationship of fungal abundance and richness with climate parameters is from Talley et al. (2002). Climate parameters are averages for the growing season (March to October, 1999). "*Sacchar.*" = *Saccharomyces.*

we collected 1 cm^2 of leaf material from each of seven haphazardly chosen plants and placed them in a sterile vial (i.e., the seven samples were combined). Leaf samples were stored at 4°C for 1–7 days before culturing. The relationship of diversity and abundance of culturable fungi with storage time was not determined. To remove fungi from leaf surfaces, we soaked and vortexed leaf samples for 15 sec in 7 ml of a sterile aqueous solution containing a 0.5% sodium chloride, 2.0% casein, and 4.0% sucrose. We spread 250 μl of the inoculated solution onto media in Petri dishes (10 cm diam.). We used three types of media all containing 2% granulated agar (Difco, Detroit, Michigan, USA): (1) potato sucrose agar [similar to potato dextrose agar except sucrose is used instead of dextrose (Dhingra and Sinclair, 1995)], (2) casein agar (for 1 L, 5 g casein, 0.5 g NaCl, and 20 g sucrose), and (3) sagebrush-leaf agar. To prepare the sagebrush-leaf agar, we removed surface secondary metabolites on 80 g of fresh sagebrush leaves by washing them in 400 ml of chloroform for 3 min and washing again in 200 ml of chloroform for 1 min. The leaf-surface secondary metabolites were not included in the medium, because they may contain antifungal properties. Leaves were placed under a hood for 1 d in order to remove chloroform and then dried at 70°C to constant weight (1–2 ds). We homogenized dried leaves in 900 ml of water and filtered the leaf extract (Whatman No. 1). We combined 500 ml of leaf extract, 250 ml of H_2O, and 250 ml of Czapek's minerals [(Dhingra and Sinclair, 1995) (1 l of Czapek's aqueous solution contained 2 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4$, and 1 ml of an aqueous solution of 1% $ZnSO_4$ and 0.5% $CuSO_4$)]. All three media contained ampicillin (60 μg/ml) to eliminate contamination by bacteria. For each site, there were two plates each of potato sucrose agar, casein agar, and sagebrush-leaf agar, giving six plates total per site. We incubated the inoculated plates at ambient temperature (approximately 22°C) for 4 d and examined them. We found little change in the number of colonies after 4 days.

Under a dissecting microscope (10–50 X), we classified colonies into morphotypes based upon colony morphology (effuse, raised, domed or umbonate), color, transparency, and shape (irregular, crenated, or rhiziod). The number of morphotypes was determined for each plate separately, a total of six values per site. We scored fungal abundance as the total number of colonies per plate. We scored fungal species richness as the total number of morphotypes per plate. We did not attempt to identify species or to compare morphotypes among plates. There was no significant effect of the culture medium on abundance or richness measures (Kruskal-Wallis $\chi^2 = 0.60$; $P = 0.74$ and $\chi^2 = 3.50$; $P = 0.17$, respectively), so the fungal abundance and richness at each site was calculated as the mean of the six plates. Fungal abundance and richness on the leaf surfaces of the most common species associated with sagebrush were estimated using similar techniques. We also collected airborne fungi by vertically swiping agar plates 10 times in the air. Results from the air and the nonsagebrush vegetation were combined

and referred to as the fungal abundance and richness of the habitat (Talley et al., 2002).

Extraction of Leaf-Surface Secondary Metabolites. From mid-June to early August of 1998, we collected two replicate samples of leaves from each sagebrush population. For each sample, we pooled approximately 20 g of leaves from each of 20 individuals for each population (a total of 400 g of leaves per population). Individuals were pooled because we found little difference in secondary metabolite composition within populations (Talley, 2001), and we were primarily interested in differences among populations.

One extract was prepared from each 400-g sample (i.e., two extracts per sagebrush population). Sagebrush secondary metabolites were extracted by gently washing intact, fresh leaves for 3 min with 10 ml of chloroform per 2 g of leaves (Kelsey, 1982). We rinsed the leaves again for 10 sec with 5 ml of chloroform per 2 g of leaves. Because the leaves and leaf cuticle were intact and the extraction rapid, we believe the extracts were primarily composed of leaf-surface metabolites. The crude extract was filtered through glass wool to remove residual leaf hairs. Chloroform in the crude extract was removed under vacuum, and the leaf-surface extract was stored at -70°C. Extracted leaves were placed under a hood for 1 d to remove chloroform and dried to constant weight at 70°C (1–2 days). We determined the investment in secondary metabolites gravimetrically as the weight of extract divided by the sum of the dry weight of leaves and extract. Investment is reported as a percent. The amount of extract per leaf-surface area was obtained by extracting leaf disks of 33 mm^2, the same size as the paper disks in the antifungal disk assay (see below). Based on several populations, the average amount of secondary metabolites on 33 mm^2 of sagebrush leaves was approximately 800 μg, and we used this weight in the bioassays. Crude extracts were redissolved in chloroform at a concentration of 200 $\mu g/\mu l$. For all assays, we used 4 μl of the 200 $\mu g/\mu l$ crude extract solution to provide 800 μg/replicate.

Fungal Test Organisms for Bioassays. The relative antifungal activity of the crude extracts from sagebrush populations was determined in growth inhibition bioassays with two nonpathogenic species of fungi. We used a laboratory yeast, *Saccharomyces cerevisiae* [JSY 999 containing three auxotrophic markers, *ura* 3-5, *his* 3Δ200, *leu* 2Δ (Hermann et al., 1997)]. We also used a *Penicillium* sp. that appeared to be the most common fungus at our study sites. In addition, a fungal survey of a salt playa in Utah listed *Penicillium* as the second most common fungus (Weber et al., 2000). Neither *Penicillium* nor *Saccharomyces* are expected to be adapted to sagebrush defenses, so they can be considered as generalized test organisms that might be sensitive to the antifungal metabolites of sagebrush. *Puccinia tanaceti* (the black rust fungus) is the most common obligate pathogen of sagebrush, but was not used as a test organism because attempts by us and others to culture it *in vitro* have never been successful.

We grew *Saccharomyces* overnight (to saturation) in YPD nutrient broth (Difco) at 30°C. To prepare test plates, we inoculated 1 L of YPD nutrient agar (Difco) cooled to 56°C with 4 ml of liquid culture. We grew *Penicillium* on YPD agar in 25 cm^2 sterile NUNC bioassay dishes (Nalge Nunc International, Naperville, Illinois, USA) for 4–5 days. Spores were suspended in a nutrient broth (Homans and Fuchs, 1970) and sprayed directly onto surface of YPD agar. YPD agar contained ampicillin (60 μg/ml) to eliminate contamination by bacteria.

Antifungal Activity of Sagebrush Secondary Metabolites. Antifungal activity of the leaf-surface extract from each sagebrush population was measured as the inhibition of fungal growth around paper disks treated with sagebrush secondary metabolites. We sterilized paper disks (Whatman No. 1, 6.5 mm diam. or 33 mm^2). For both *Saccharomyces* and *Penicillium* assays, we infused three replicate disks with 4 μl of crude extract for each of the two extracts from every sagebrush population, giving six measures per sagebrush population per assay. The chloroform was evaporated in a laminar flow hood leaving 800 μg of leaf-surface extract per disk. The negative control was solvent-treated filter paper (4 μl of chloroform). For the *Saccharomyces* assay, we poured 100 ml of inoculated agar over test disks that had been aseptically glued with sterilized 2% agar to the bottom of 25 cm^2 sterile NUNC bioassay dishes (Nalge Nunc International). For the *Penicillium* assay, the infused disks were placed on the top of the inoculated agar. Plates were incubated at 30°C for 2 days. Using calipers, we measured the annulus around the disk that lacked fungal growth (the zone of inhibition). We noted the strength of inhibition visually by the clarity of the zone of inhibition. Clear zones were given a score of 1.0, zones that had little growth were scored 0.5, and zones with a lot of growth were scored 0.25. Antifungal activity per weight of secondary metabolites (800 μg) was calculated by multiplying the width of the zone by the strength of the inhibition. Because sagebrush populations varied in the amount of secondary metabolites per dry weight of the leaf, we calculated a second parameter, the total antifungal activity (activity per gram of leaf), by taking the product of antifungal activity per weight of secondary metabolites (activity per 800 μg of secondary metabolites) and secondary metabolite investment (grams of metabolites per grams of leaf).

Bioautography and Detection of Antifungal Secondary Metabolites. We used bioautography to determine which of the secondary metabolites in a crude extract were antifungal. In bioautography, compounds are separated by thin-layer (TL) chromatography, and TL chromatograms are overlaid with medium inoculated with a test organism. Areas lacking fungal growth can then be matched to particular areas on the TL chromatograms. In this study, we used two test organisms, *Saccharomyces* and *Penicillium*. Using three replicates of each of the two leaf extracts per sagebrush populations (i.e., six measures per population), we separated secondary metabolites in 800 μg of crude extract by TL chromatography (20 $\times$ by 20-cm aluminum-backed silica gel plates 60 $WF_{254}S$; EM Science, Gibbstown,

New Jersey, USA). We used a solvent system of chloroform–petroleum ether–ethyl acetate 2:2:1 v/v to separate the leaf-surface secondary metabolites (Kelsey et al., 1976). The residual solvent on the TL chromatograph was allowed to evaporate in a biohazard laminar flow hood. The total number of spots was measured as those that were visible under ultraviolet radiation (UV-B, 254 nm and UV-A, 365 nm) and were revealed by sulfuric acid. The 254-nm radiation detected metabolites that absorb at 254 nm and the 365-nm radiation detected metabolites that absorb at 365 nm and that also fluoresce efficiently. To obtain the position of the antifungal secondary metabolites, we placed TL chromatograms in sterile NUNC bioassay dishes. For *Saccharomyces* bioautography, we overlaid TL chromatograms with 40 ml of inoculated YPD nutrient agar. For *Penicillium* bioautography, we sprayed a suspension of spores in a nutrient broth onto the TL chromatogram (Homans and Fuchs, 1970). All media contained ampicillin (60 μg/ml) to eliminate contamination by bacteria. Bioautographs were incubated at 30°C for 2 days. The areas lacking fungal growth (*Saccharomyces*) or spore germination (*Penicillium)* corresponded to secondary metabolites that were antifungal. To record the activity, we overlaid bioautographs with transparencies, traced the areas that lacked fungal growth, and measured the position of the antifungal spots relative to the origin (R_f).

To determine the number of antifungal spots for each population, we considered a spot as present (value = 1) if it occurred in any of the replicates or absent if it occurred in none (value = 0). To compare the number of antifungal spots to the number of visible secondary metabolites, we recorded all spots on the TL chromatograms when viewed under UV and when developed with concentrated sulfuric acid followed by heating for 2 min. The number and position of secondary metabolites were recorded with color photocopies and digital photographs. Our estimates of the number of antifungal secondary metabolites may be conservative, because TLC separates secondary metabolites by polarity, and several antifungal secondary metabolites can comigrate forming a single antifungal spot. Furthermore, secondary metabolites that degrade after extraction and those that work synergistically may not be detectable.

Alternative Functions for Antifungal Secondary Metabolites. We examined whether the antifungal secondary metabolite spots on the bioautograph have alternative functions such as protection from ultraviolet light or oxidation. To determine whether spots on the bioautograph also absorb UV radiation, we visually compared the location of spots absorbing at 254 nm and fluorescing at 365 nm with the location of the antifungal spots.

We determined whether the antifungal spots on the bioautograph could also function as antioxidants. To determine which spots on the bioautograph were antioxidant, we sprayed TL chromatograms with an oxidant solution of 9.0 mg β-carotene (all reagents from Sigma, St. Louis, Missouri, USA) in 30 ml of chloroform, 60 ml of ethanol, and two drops of linoleic acid (Pratt and Miller, 1984). There were three experiments per extract and two extracts per population for a total of six

measures per sagebrush population. We used 200 μg of DL-α-tocopherol acetate and 200 μg of ascorbic acid as positive controls. Plates were placed under an UV-B radiation emitting light and observed for bleaching. Spots on the TL chromatogram that remained orange corresponded to antioxidant secondary metabolites. The positions of the antioxidant secondary metabolites were sketched on the plates with a pencil. Plates were examined every 30 min for 3 hr, and we scored the antioxidant strength of the compounds based upon the length of the time that spots remained orange. In particular, we scored the antioxidant strength of the regions of the TL chromatograph that also showed antifungal activity in the bioautographs with the two fungi.

Soil Analyses. We measured soil properties that might influence plant vitality. To control for variation in soil nutrients due to seasonal fluctuations, we collected soil from 22 sites within a month during the spring of 1999. We discarded the top 5 cm of soil and collected 2–3 kg of soil up to a depth of 20 cm. We dried soil samples for 48 hr or until constant weight at 105°C. We determined the percent silt and clay with a hydrometer. We determined soil pH using 15 g of ground soil in 30 ml of distilled water. To determine percent organic carbon and nitrogen of soil by mass spectroscopy, we first removed the inorganic carbon by bringing soil samples to a pH between 4 and 5 with 5N HCl. Soil samples were then flash combusted on an elemental analyzer (Carlo Erba, Milan, Italy) coupled to an isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany) operated in continuous flow mode.

Fungal Disease. The most common disease-causing organism on sagebrush in our study was *Puccinia tanaceti*, an obligate pathogen of sagebrush. All spore stages of *P. tanaceti* are reported to occur on one host (Welch and Nelson, 1995), and we are unaware of reports of alternative hosts for *P. tanaceti* on species that cooccur with sagebrush. *P. tanaceti* produces characteristic pustules from sporulation of the black telial sori on the leaf surfaces (Welch and Nelson, 1995). Identification of *P. tanaceti* on leaf specimens was confirmed by D. L. Nelson (personal communication). During October 1999, we scored 25 individuals in each sagebrush population for *P. tanaceti* disease. The fall is the best time of year to score sagebrush plants visually for the presence of *P. tanaceti* disease because that is when the telial sori are present on the persistent leaves (Welch and Nelson, 1995). We chose plants in a population by generating random numbers that corresponded to the distance a plant was along a transect through the population. Disease is commonly reported in two ways: incidence, which is the proportion of individuals in a population that are diseased, and severity, which is the degree to which the individuals in a population are diseased. Because sagebrush has small leaves and diseased leaves generally have multiple pustules, we measured the severity of the disease by examining the entire plant and estimating the percent of diseased leaves on a plant as 0, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100%.

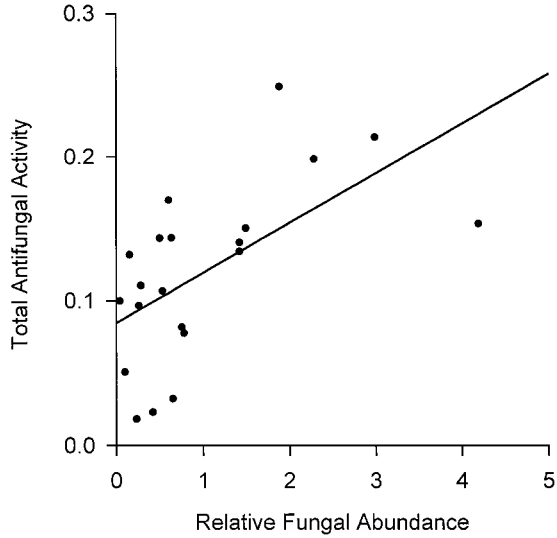


FIG. 2. Total antifungal activity of sagebrush secondary metabolites against *Saccharomyces* as a function of the fungal abundance in the habitat ($r^2 = 0.36$, $P < 0.004$; antifungal activity $= 0.08 + 0.03 \times$ fungal abundance). The units for total antifungal activity are millimeters times grams of secondary metabolites divided by the leaf dry weight where millimeters refer to the width of the zone of inhibition.

Statistical Analyses. We performed univariate and multivariate statistical analyses in SYSTAT, Version 7 (Wilkerson, 1988) and JMP (SAS Institute Inc., Cary, North Carolina, USA). For differences among populations and moisture categories, we applied Kruskal-Wallis nonparametric analysis. Using simple linear regression, we analyzed antifungal activity or the number of antifungal secondary metabolites as a function of fungal abundance and richness and climate parameters.

RESULTS

Relationship of Antifungal Activity with Fungal Prevalence in Habitat and with Climate. We found greater total antifungal activity of sagebrush leaf-surface secondary metabolites in habitats with greater fungal abundance (Table 1; Figure 2; Appendices A and B). Fungal abundance explained 36% and 37% of the variance in antifungal activity in the *Saccharomyces* and *Penicillium* bioassays, respectively. Fungal abundance in the habitat was also positively related to the antifungal activity per weight of metabolites, explaining 29% of the variance in the *Saccharomyces* bioassays and 30% in the *Penicillium* bioassays (Table 1). Even though *Saccharomyces* (Hemiascomycetes) and *Penicillium* (Euascomycetes) are not closely

related fungi, the total activity of secondary metabolites against *Saccharomyces* and *Penicillium* were highly correlated ($r = 0.995$, $P < 0.001$), suggesting broad-spectrum, antifungal activities.

The climate variables that were most highly correlated with the fungal abundance and richness of the habitat (Talley et al., 2002) also were good predictors of total antifungal activity of sagebrush secondary metabolites (Figure 3; Table 1). The climate variable that explained the most variance in total antifungal activity among sagebrush populations was the average VPD, a measure of the desiccating capacity of the air (for example, for total antifungal activity against *Saccharomyces*, $r^2 = 0.60$, $P < 0.001$; Figure 3a; Table 1). A climate variable expressing the duration of unfavorable conditions to fungi, the average number of consecutive days with maximum diurnal relative humidity <50%, was the best predictor of fungal abundance and richness in the habitat (Table 1) (Talley et al., 2002). This climate measure also was negatively related to the total antifungal activity of the sagebrush secondary metabolites against *Saccharomyces* (Figure 3b) and *Penicillium* (Table 1). Similar relationships were found for climate variables and antifungal activity per weight of metabolites (Table 1).

Composition and Number of Antifungal Secondary Metabolites. The bioautograph was divided into six regions, and each region was scored for antifungal secondary metabolites (see Appendix C). Because the regions were broadly defined, the "same" spot from different populations may contain different secondary metabolites that have similar polarities. The number of antifungal secondary metabolites ranged from 0 to 6 among sagebrush populations, and most populations had a unique combination of active spots (Appendix C). Spot 1 was the most common antifungal spot on the *Penicillium* bioautographs, found in 21 of 26 sagebrush populations, but was present in only 3 of 23 sagebrush populations in the *Saccharomyces* bioautographs. Spot 5 was the most common in the *Saccharomyces* bioautographs, found in 12 of 23 sagebrush populations, but also occurred in the *Penicillium* bioautographs (14 of the 26 populations). It is unlikely that among-population variation was due to individual variation within a population. In a related study, we found that most secondary metabolites characteristic of each population were shared by most or all of the individuals within that population (Talley, 2001). Plasticity in response to environmental variation was not investigated. We also found that fungal abundance in the habitat was positively related to the number of antifungal spots in the *Saccharomyces* bioautography ($r^2 = 0.34$, $P < 0.01$; Figure 4), but not the *Penicillium* bioautography ($r^2 = 0.12$, $P = 0.10$).

We found no relationship between the number of antifungal spots and the total number of detectable spots, indicating that increased activity and diversity of antifungal secondary metabolites was not a simple reflection of an increase in the total diversity of secondary metabolites ($r = 0.20$, $P = 0.55$ for *Penicillium*; $r = 0.24$, $P = 0.25$ for *Saccharomyces*). The number of antifungal spots on the bioautographs was highly correlated with the antifungal activity per mass of

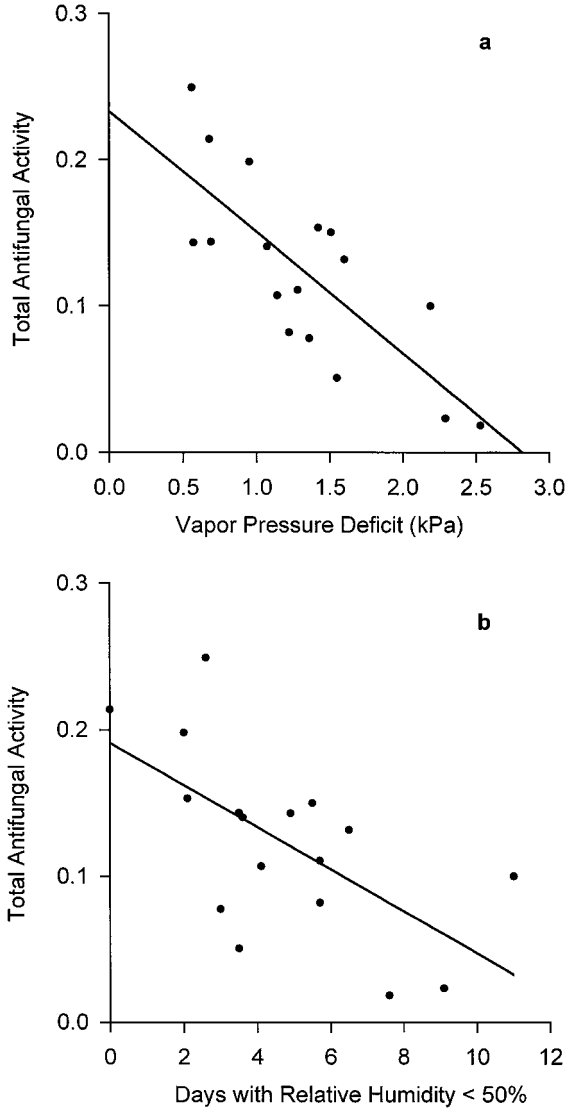


FIG. 3. (**a**) Total antifungal activity of sagebrush secondary metabolites against *Saccharomyces* as a function of the average VPD of the sagebrush habitats ($r^2 = 0.60$, $P < 0.001$; antifungal activity $= 0.23 - 0.08 \times$ the average VPD). (**b**) Total antifungal activity of sagebrush secondary metabolites against *Saccharomyces* as a function of the average number of consecutive days with maximum diurnal relative humidity less than 50% (arh50) ($r^2 = 0.40$, $P < 0.006$; antifungal activity $= 0.19 - 0.01 \times$ arh50). The units for total antifungal activity are millimeters times grams of secondary metabolites divided by the leaf dry weight where millimeters refer to the width of the zone of inhibition.

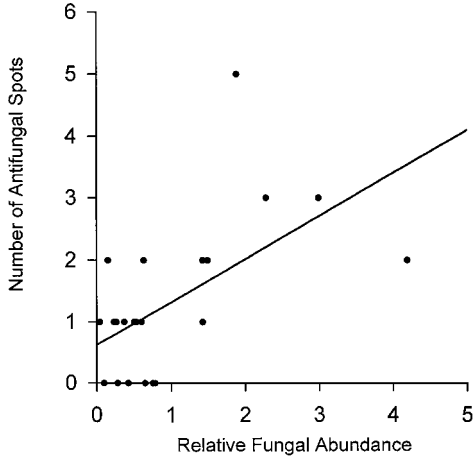


FIG. 4. The number of antifungal spots from *Saccharomyces* bioautography as a function of the fungal abundance in the habitat ($r^2 = 0.34$, $P < 0.005$; number of antifungal spots $= 0.62 + 0.69 \times$ fungal abundance).

metabolites for both *Saccharomyces* and *Penicillium* ($r = 0.85$, $P < 0.001$ and $r = 0.68$, $P < 0.001$, respectively), indicating that the number of antifungal metabolites also contributes to antifungal activity.

Investment in Leaf-Surface Secondary Metabolites. Investment in leaf-surface secondary metabolites averaged 17% (± 2%) of total leaf dry weight, ranging from 12% to 22% across sagebrush populations (Appendix A). We found no relationship between investment in secondary metabolites and fungal abundance in the habitat ($r = 0.17$, $P = 0.41$). The investment in secondary metabolites was not related to climate parameters such as VPD ($r = 0.32$, $P = 0.19$) nor the average number of consecutive days with maximum diurnal relative humidity <50% ($r = 0.33$, $P = 0.18$). We also found no relationship between investment in secondary metabolites and the number of detectable compounds ($r = 0.01$, $P = 0.97$), the antifungal activity per weight of metabolites in the *Saccharomyces* assay ($r = 0.39$, $P = 0.08$), or the number of antifungal compounds in the *Saccharomyces* bioautography ($r = 0.04$, $P = 0.82$). Thus, there was no relationship between the investment in secondary metabolites and either the activity or the diversity of secondary metabolites. The significant positive relationship between total antifungal activity and fungal abundance was, therefore, not due to greater investments in secondary metabolites, but due to greater activity per gram of metabolite.

Alternative Functions for Antifungal Secondary Metabolites. We found no correlation between the number of antifungal spots on the bioautograph and the number of spots absorbing UV light at 254 nm and 365 nm, the number of antioxidant spots, or the strength of the antioxidant spots on the TL chromatograms

of sagebrush secondary metabolites (either for *Saccharomyces* or *Penicillium*, Appendix C). Furthermore, we found no significant relationships between the number of UV absorbing and antioxidant spots or the strength of the antioxidant spots with investment in secondary metabolites, climate variables, geographic location, or fungal abundance and richness.

Relative Fungal Abundance and Richness on Sagebrush Vegetation. The fungal abundance in the habitat was positively correlated with fungal abundance on sagebrush leaves ($r = 0.47$, $P < 0.03$), and the fungal richness in the habitat was positively correlated with the fungal richness on sagebrush leaves ($r = 0.58$, $P < 0.005$). Fungal abundance and richness on the sagebrush leaves were positively correlated ($r = 0.62$, $P < 0.003$).

For *Saccharomyces*, fungal abundance on sagebrush leaves was positively correlated with both the antifungal activity per weight of metabolites and the number of antifungal secondary metabolites ($r = 0.50$, $P < 0.03$; $r = 0.54$, $P < 0.02$, respectively). For *Penicillium*, fungal abundance on sagebrush leaves was positively correlated with the antifungal activity per weight of metabolites ($r = 0.50$, $P < 0.04$), but not the number of antifungal secondary metabolites ($r = 0.17$, $P = 0.44$). There was no relationship between fungal abundance on sagebrush leaves and the total antifungal activity for *Saccharomyces* and *Penicillium*. Fungal abundance in the habitat, assayed as airborne fungi and from leaves of nonsagebrush species, generally had stronger relationships with antifungal activity and climate parameters than did fungal abundance on sagebrush leaves. The leaf-surface antifungal secondary metabolites of sagebrush may eliminate many fungi. Hence, fungal abundance in the habitat may better measure fungal abundance than fungal abundance on sagebrush leaves.

Relationship of Antifungal Activity and Metabolites with Soil Characteristics. We found positive relationships between soil organic carbon and nitrogen and antifungal activity per weight of metabolites in the *Saccharomyces* assay ($r = 0.68$, $P < 0.001$; $r = 0.75$, $P < 0.001$, respectively) and in the *Penicillium* assay ($r = 0.67$, $P < 0.001$; $r = 0.74$, $P < 0.001$, respectively). We also found positive relationships between soil organic carbon and nitrogen and the number of antifungal metabolites in the *Saccharomyces* ($r = 0.65$, $P < 0.001$; $r = 0.69$, $P < 0.001$, respectively) and the *Penicillium* ($r = 0.45$, $P < 0.05$; $r = 0.57$, $P < 0.01$, respectively) bioautographies. Antifungal activity and metabolites did not correlate with soil pH, sand, silt, or clay.

Fungal Disease on Sagebrush. We examined rust disease caused by the obligate fungal pathogen, *Puccinia tanaceti* DC, (Appendix A) as a function of fungal abundance in the habitat, climate variables, and antifungal activity of sagebrush secondary metabolites. Fungal abundance in the habitat positively correlated with variation in rust incidence ($r = 0.42$, $P < 0.03$) and severity ($r = 0.40$, $P < 0.05$) among sagebrush populations even though *P. tanaceti* was not cultured by our fungal census methods. We found no correlation of rust on sagebrush either

with climate or with antifungal activity against *Saccharomyces* or *Penicillium* when variables were considered singly. However, using multiple regression, total antifungal activity combined with the fungal abundance in the habitat explained a significant portion of the variance among populations ($P < 0.05$) for rust incidence ($r^2 = 0.25$ for *Penicillium*, $r^2 = 0.25$ for *Saccharomyces*) and severity ($r^2 = 0.30$ for *Penicillium*, $r^2 = 0.30$ for *Saccharomyces*), suggesting that disease depends on both plant defenses and fungal prevalence.

Comparisons among Habitat Types. To compare potential selection gradients and sagebrush responses among habitats, we divided sagebrush populations into classes of low and high available moisture (based on the VPD of the sagebrush habitat, Figure 5). Sites with high available moisture had a greater fungal abundance in the habitat than sagebrush habitats with low available moisture

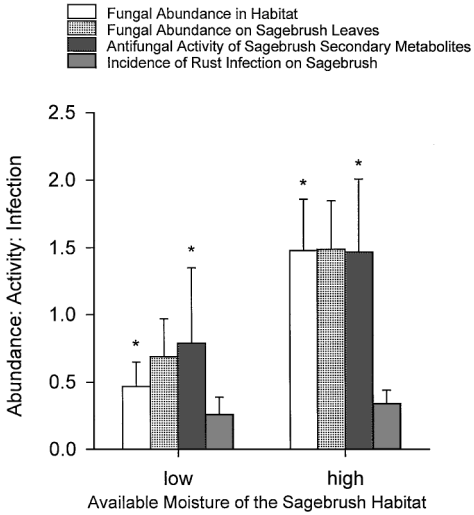


FIG. 5. Fungal abundance in the habitat, fungal abundance on sagebrush leaves, total antifungal activity of sagebrush secondary metabolites against *Saccharomyces*, and incidence of *P. tanaceti* diseases on sagebrush in habitats with high or low moisture availability. Sagebrush populations were divided into low and high available moisture groups based on the VPD of the sagebrush habitat (low moisture = 2.53 to 1.51 kPa, $N = 8$ sites; high moisture = 1.42 to 0.56 kPa, $N = 11$ sites). Fungal abundance is a normalized measure of the number of fungal colonies per plate from both air and vegetation sampling (Talley, 2001). The units for total antifungal activity are mm x g secondary metabolites divided by the leaf dry weight where mm refers to the width of the zone of inhibition. The values for total antifungal activity were multiplied by 10 to make them visible on the figure. Incidence is the proportion of infected individuals within a population. *Statistically significant ($P < 0.05$) differences between sagebrush habitats with high and low available moisture.

(Kruskal-Wallis, $\chi^2 = 5.73$, $P = 0.02$). We found a nonsignificant trend for the fungal abundance and richness on sagebrush leaves ($\chi^2 = 1.71$, $P = 0.19$; $\chi^2 = 0.12$, $P = 0.72$, respectively). Sagebrush populations with high available moisture had greater total antifungal activity of secondary metabolites against *Saccharomyces* ($\chi^2 = 4.04$, $P = 0.04$) and *Penicillium* ($\chi^2 = 4.04$, $P = 0.04$) than sagebrush habitats with low available moisture. We found no difference between low and high moisture classes in the incidence ($\chi^2 = 1.12$, $P = 0.29$) or severity ($\chi^2 = 1.03$, $P = 0.31$) of rust disease caused by *P. tanaceti*.

DISCUSSION

Sagebrush populations are broadly distributed among a range of habitats and climates and may, therefore, have adapted to the local abiotic and biotic selective pressures. Although summer drought is considered to be the most important stress in the region dominated by sagebrush, we propose that pathogens also may be important selective agents. Fungi are mainly limited by moisture and temperature (Cooke and Whipps, 1993; Harrison et al., 1994; Jones, 1998) and are more abundant and speciose in sage habitats with high relative humidity and moderate temperatures than in drier, hotter habitats (Talley et al., 2002). Our data suggest that sagebrush populations that occur in a broad range of habitats experience different levels of selection by fungi, and correspondingly produce different profiles of antifungal secondary metabolites. Furthermore, the primary location of those compounds on the leaf surface is consistent with their role as a defense mechanism against fungi.

Sagebrush makes a substantial investment in leaf-surface metabolites, averaging 17% of dry weight [similar results were found by Kelsey et al. (1983)]. We found that the total antifungal activity of leaf-surface secondary metabolites was positively related to fungal prevalence in the habitat and climate variables favorable to fungi (Table 1, Figure 2). However, the investment in all leaf-surface secondary metabolites (grams of metabolites per grams dry weight of the leaf) was not correlated with fungal prevalence in the habitat or climate. Nor was the investment in all secondary metabolites correlated with either the antifungal activity per weight of metabolites or with the number of antifungal secondary metabolites. These results suggest that most leaf-surface secondary metabolites have little or no antifungal activity. Hence, investment in all secondary metabolites *per se* is not a good measure of sagebrush defense against fungi.

Instead, differences in total antifungal activity among sagebrush populations were due to differences in the toxicity of metabolites to fungi and in secondary metabolite composition. Sagebrush populations in mesic habitats with climates favorable to fungi had greater activity per grams of metabolite and a greater number of antifungal spots as compared to sagebrush populations in habitats with climates

unfavorable to fungi (Table 1). The fact that these results were obtained in assays with fungi from two families of ascomycetes suggests that sagebrush secondary metabolites may function as a defense against a variety of fungi.

Our data are consistent with the hypothesis that plant enemies select for both elevated levels of defense as well as a greater diversity of defensive secondary metabolites. More antifungal secondary metabolites may evolve in plants of high-risk habitats in order to balance the asymmetries in plant–pathogen interactions. In other words, the large effective population sizes and short generation times of pathogens could lead to rapid changes in the species composition of the pathogen community and in gene frequencies within a population of pathogens (May and Anderson, 1990). Under such conditions, multiple antifungal secondary metabolites may provide more effective defense against fungal pathogens. Elucidation of the structure of sagebrush antifungal secondary metabolites may also provide a test of the hypothesis that more active compounds are more biosynthetically derived (Berenbaum, 1978).

Soil nitrogen and organic carbon (but not soil pH or texture) are positively correlated with antifungal activity and metabolites. Although environmental interactions may affect the production of secondary metabolites, we found that sagebrush grown from seeds in a greenhouse retained the secondary metabolite composition and antifungal characteristics of their parent populations (Talley, 2001). Hence, correlation of the soil with antifungal compounds may not be indicative of plant environmental responses, and the differences in antifungal secondary metabolites among sagebrush populations probably have a genetic basis.

Our evidence for a genetic basis of antifungal secondary metabolites as well as for a correlation between antifungal activity and fungal prevalence in the habitat suggests that variation in leaf-surface metabolites may be a consequence of differential selection by fungal pathogens. However, with correlative studies it is difficult to eliminate the possibility that a third factor, which covaries with fungal abundance, may be the real selective agent. For example, antifungal secondary metabolites may have alternative functions, such as UV or oxidation protection, reflection of light, and defense against other sagebrush enemies. Although it is impossible to rule out all alternative functions, we evaluated several of the most likely abiotic functions. One would expect that the UV-B absorbing and antioxidant potential of secondary metabolites would correlate with UV flux as influenced by elevation and latitude. However, we found no such relationship of UV absorbing potential and antioxidant potential with climate or any geographic parameters. There was also no relationship of the number of UV absorbing spots and antioxidant spots with either antifungal activity, the number of secondary metabolites, or fungal abundance and richness in the habitat. Trichomes (and their contents) may function by reflecting light, but this would predict more trichomes in arid habitats (Ehleringer and Mooney, 1978). Instead, we found no evidence for an effect of climate on the investment in leaf-surface secondary metabolites. Thus, we

found no evidence that the antifungal secondary metabolites are serving alternative functions against abiotic stress that would create confounding selection gradients among our study sites.

Our results also suggest that herbivores are probably not important selective agents driving the geographic variation we found in the antifungal secondarymetabolites of sagebrush. If herbivores were important, one might predict a general response for most of the secondary metabolites, but in our study, only the small subset of metabolites (six out of 44) that were antifungal showed a correlation with fungal abundance. The specificity of this response suggests that fungi, rather than other selective agents, are probably the main selective agent influencing the distribution of bioactive secondary metabolites in sagebrush.

Another requirement for demonstrating natural selection is to show that differences in antifungal secondary metabolites lead to differences in plant fitness. As a partial test, we examined the incidence of rust disease caused by the most obvious pathogen on sagebrush, *Puccinia tanaceti*. *P. tanaceti* is an obligate pathogen on sagebrush and can cause a 10-fold reduction in seed production (Welch and Nelson, 1995). Given the potential negative impact on fitness, sagebrush should be selected to have defenses against *P. tanaceti.* We found that rust incidence and severity were not correlated with climate variables and were similar among populations from dry and moist habitats (Figure 5). We suggest that this arises because rust disease is a function of both the favorability of the habitat for fungi and of the defense afforded by leaf-surface secondary metabolites. For example, in hot, dry habitats, sagebrush invests little in antifungal defenses, but harsh abiotic conditions reduce fungal abundance, leading to reduced rust incidence and severity. In contrast, in moist, cool habitats, where there is high fungal abundance and high investment in secondary metabolites by sagebrush, plant defense metabolites have reduced rust incidence and severity. Thus, rust incidence may result from a balance between the prevalence of *P. tanaceti* in the environment and the effectiveness of antifungal defenses in the host plants. Censusing *P. tanaceti* over multiple years, combined with common garden experiments, would provide a test of this hypothesis. We cannot definitively affirm that disease incidence in our study populations is independent of other plant defenses (i.e., leaf toughness, trichome density) because we did not assess variation in the plant defenses.

An alternative interpretation of our results is that rust incidence and severity do not vary geographically because (1) *P. tanaceti* is not sensitive to the same antifungal secondary metabolites as *Saccharomyces* and *Penicillium*, or (2) fungal prevalence does not reflect *P. tanaceti* abundance. Because it was not possible to culture *P. tanaceti* from spores, we could not conduct bioassays to measure toxicity of metabolites against *P. tanaceti.* However, two pieces of evidence suggest that the distribution of *P. tanaceti* across habitats mirrors our measure of fungal prevalence. First, rust was positively correlated with fungal abundance ($r = 0.42$, $P < 0.03$),

suggesting that *P. tanaceti* abundance may vary with environmental conditions in a manner parallel to the other fungi in our study. Second, other *Puccinia* species have moisture and temperature optima that are similar to most mesophils and are consistent with preferences for moist sites (Burrage, 1970; de Vallavielle-Pope, 1995; Hau and de Vallavielle-Pope, 1998; Geagea et al., 1999). Thus, it seems likely that *P. tanaceti* is more prevalent in moist sites but that rust incidence is kept low by effective antifungal metabolites.

The qualitative and quantitative differences in secondary metabolites among sagebrush populations suggest not only that selective pressures by fungal pathogens differ among populations, but also that plant responses to selection represent independent solutions. If all populations in sites with high fungal prevalence had similar secondary metabolites, one might conclude that effective defenses had arisen once and were maintained as the well-defended genotypes spread into similar habitats. This result may be the case for two high-altitude populations of sagebrush that are separated by less than 13 km, Racetrack and Strawberry. Both have highly active extracts with antifungal spots that are similar in number and intensity (Appendix C). However, our data suggest that, although populations from fungal-rich habitats are all well defended, many have arrived at unique solutions, presumably by independent evolutionary trajectories. For example, three of the populations with the most highly antifungal extracts (Emigration, La Sal, and Racetrack) differed in the number of antifungal secondary metabolites, and even shared spots varied in strength (Appendix C). Similarly, when comparing three adjacent populations that differ in altitude but are less than 6 km apart (Tintic High, Tintic Low, and Tintic Wash), the number and activity of secondary metabolites also differed, following trends predicted by fungal abundance (Appendix C). Thus, related populations that appear to differ in potential selective pressures imposed by fungi and in their defensive secondary metabolites likely have independently derived different suites of antifungal defenses.

In summary, much remains to be learned about the evolutionary paradigm of how secondary metabolite defenses arise and are maintained in plants. Consequently, the evolution of plant secondary metabolites due to selection by herbivores and pathogens currently is an area of great interest (Marquis and Alexander, 1992). Nevertheless, the role of pathogens in driving the evolution of secondary metabolites is little understood. To our knowledge, we report the first intraspecific evidence relating the activity and number of antifungal secondary metabolites to fungal prevalence among a large number of natural plant populations. Despite complications in showing that a particular secondary metabolite evolved in response to a particular plant enemy, the relationship between estimated attack levels by plant enemies and the level and diversity of antifungal secondary metabolites provides strong correlative evidence that fungal pathogens have selected for plant chemical defense.

APPENDIX A. LOCATION, FUNGAL PREVALENCE OF HABITAT, RUST, AND INVESTMENT IN SAGEBRUSH SECONDARY METABOLITES[a]

Site No.	Site Name	Elevation (m)	Latitude	Longitude	Fungal abundance in habitat	Rust incidence	Rust severity	Secondary metabolite investment (% dry weight)
1	Bramble	2227	37°40′11″	111°48′20″	0.99	0.68	3.90	15.2
2	Canyonlands	1442	38°38′44″	109°45′04″	0.75	0.56	1.83	17.9
3	Duchesne	1633	40°10′34″	110°06′16″	1.49	0.83	9.14	19.8
4	Emigration	1715	40°45′47″	111°42′36″	2.28	0.53	2.51	18.5
5	Farmington	1433	40°59′46″	111°53′05″	4.19	0.86	14.65	19.9
6	Francis Peak	2900	41°01′53″	111°50′20″	0.50	0.06	0.84	16.7
7	GE Slot	2045	37°52′08″	111°06′37″	0.53	0	0	14.9
8	Grand Escalante	1726	37°51′40″	111°17′53″	0.37	0.56	12.98	nd
9	Gunlock Res.	1030	37°10′46″	113°44′51″	0.23	0	0	16.8
10	Kodachrome Basin	1831	37°30′05″	111°59′37″	0.28	0	0	19.1
11	La Sal	2554	38°23′38″	109°09′52″	0.63	0.22	2.38	14.7
12	Leeds	1290	37°16′12″	113°22′37″	0.42	0	0	16.7
13	Pintura	1285	37°19′16″	113°16′46″	0.04	0	0	19.3
14	Racetrack	3113	40°18′42″	111°08′14″	1.88	0.08	0.54	15.3
15	Strawberry	2245	40°12′35″	111°11′13″	2.99	0.19	0.19	15.3
16	Tintic High	2027	39°57′35″	112°05′16″	1.42	0.28	0.16	15.6
17	Tintic Low	1547	39°56′53″	112°00′47″	0.10	0	0	15.9
18	Tintic Wash	1649	39°57′23″	112°02′44″	0.78	0.98	13.17	15.9
19	Wendover	1640	40°28′59″	114°09′20″	0.15	0	0	18.8
20	Littlefield	1220	37°05′09″	113°51′17″	0.60	0	0	17.0
21	La Loop	1677	38°27′10″	109°23′52″	0.16	0.08	0.16	12.3
22	Needles	1984	38°03′14″	109°24′34″	0.17	0.64	2.51	13.4
23	Little Sahara	1576	39°43′09″	112°13′05″	0.26	0.04	0.02	19.8
24	Stansbury	1654	40°34′26″	112°29′05″	0.65	0.24	0.04	21.8
25	Dugway Gate	1350	40°10′50″	113°06′29″	1.42	0	0	20.3
26	Dugway Camp	1350	40°12′50″	113°06′29″	nd	nd	nd	17.8

[a] Fungal abundance is a normalized measure of the number of fungal colonies per plate from both air and vegetation sampling (Talley et al., 2002). Other parameters are defined in the text. Missing data are noted by "nd."

APPENDIX B. ANTIFUNGAL ACTIVITY AND NUMBER OF SAGEBRUSH SECONDARY METABOLITES[a]

Site No.	Site Name	Total anti-fungal activity against *Saccharomyces*	No. of anti-fungal spots from *Saccharomyces* bioautography	Total anti-fungal activity against *Penicillium*	No. of antifungal spots from *Penicillium* bioautography	No. of visible secondary metabolites under UV radiation (254 and 365 nm)	No. of secondary metabolites when developed with sulfuric acid	Total no. of secondary metabolites
1	Bramble	nd	nd	nd	1	3	7	10
2	Canyonlands	0.08	0	0.08	2	5	6	11
3	Duchesne	0.15	2	0.15	5	5	9	14
4	Emigration	0.20	3	0.20	5	5	12	17
5	Farmington	0.15	2	0.15	3	4	8	12
6	Francis Peak	0.14	1	0.14	3	3	5	8
7	GE Slot	0.11	1	0.12	2	10	12	22
8	Grand Escalante	nd	1	nd	3	5	6	11
9	Gunlock Res.	0.02	1	0.02	0	2	9	11
10	Kodachrome Basin	0.11	0	0.11	4	5	6	11
11	La Sal	0.14	2	0.14	4	7	6	13
12	Leeds	0.02	0	0.02	2	6	7	13
13	Pintura	0.10	1	0.10	3	6	3	9
14	Racetrack	0.25	5	0.23	5	6	8	14
15	Strawberry	0.21	3	0.21	6	8	7	15
16	Tintic High	0.14	2	0.14	2	4	2	6
17	Tintic Low	0.05	0	0.05	3	6	8	14
18	Tintic Wash	0.08	0	0.08	3	4	7	11
19	Wendover	0.13	2	0.13	3	3	7	10
20	Littlefield	0.17	1	0.17	5	6	4	10
21	La Loop	nd	nd	nd	3	2	6	8
22	Needles	nd	nd	nd	4	1	5	6
23	Little Sahara	0.10	1	0.10	3	4	7	11
24	Stansbury	0.03	0	0.03	4	4	6	10
25	Dugway Gate	0.13	1	0.13	2	2	8	10
26	Dugway Camp	0.08	1	0.08	2	3	7	10

[a] Missing data are noted by an “nd”.

APPENDIX C. AVERAGE ACTIVITY OF SIX ANTIFUNGAL SPOTS ON *Saccharomyces* AND *Penicillium* BIOAUTOGRAPHS[a]

	Position of antifungal spot on *Saccharomyces* bioautograph						Position of antifungal spot on *Penicillium* bioautograph					
Antifungal spot	1	2	3	4	5	6	1	2	3	4	5	6
R_f begin	0.08	0.20	0.31	0.41	0.51	0.71	0.08	0.20	0.31	0.41	0.51	0.71
R_f end	0.19	0.30	0.40	0.50	0.70	0.90	0.19	0.30	0.40	0.50	0.70	0.90
Bramble	nd	nd	nd	nd	nd	nd	0.08	0	0	0	0	0
Canyonlands	0	0	0	0	0	0	0	0.05	0	0	0.13	0
Duchesne	0	0	0.04	0	0.50	0	0.50	0.09	0.05	0.01	0.10	0
Emigration	0	0	0.21	0.04	0.04	0	0.46	0.32	0.07	0.14	0.15	0
Farmington	0	0.27	0	0	0.44	0	0.61	0.73	0	0	0.26	0
Francis	0	0.67	0	0	0	0	1	1	0.09	0	0	0
Grand Escalante	0	0	0	0	0.38	0	0	0	0.07	0.03	0	0
GE Slot	0	0	0	0	0.08	0	0	0	0.08	0	0.01	0.06
Gunlock	0	0.04	0	0	0	0	0	0	0	0	0	0
Kodachrome	0	0	0	0	0	0	0	0	0.05	0.11	0.01	0.06
La Sal	0	0	1	0	0.50	0	0.10	0.05	0.55	0	0.10	0
Leeds	0	0	0	0	0	0	0.13	0	0.04	0	0	0
Pintura	0	0	0	0	0.50	0	0.21	0	0.13	0	0.01	0
Racetrack	0.25	0	0.08	1	1	0.60	0.75	0.33	0.50	0.08	0.50	0
Strawberry	0.50	0	0	1	0.83	0	0.73	0.36	0.30	0.11	0.12	0.07
Tintic High	0	0.38	0	0	0.04	0	0.50	1	0	0	0	0
Tintic Low	0	0	0	0	0	0	0.21	0.01	0.01	0	0	0
Tintic Wash	0	0	0	0	0	0	0.13	0.04	0.04	0	0	0
Wendover	0.08	0	0	0	0.50	0	0.13	0	0.05	0	0.08	0
Littlefield	0	0	0	0	1	0	0.25	0.06	0.19	0	0.02	0.01
La Loop	nd	nd	nd	nd	nd	nd	0.01	0.01	0	0.03	0	0
Needles	nd	nd	nd	nd	nd	nd	0.17	0.09	0	0.01	0.02	0
Sahara	0	0	0	0.08	0	0	0.21	0.18	0.04	0	0	0
Stansbury	0	0	0	0	0	0	0.01	0.13	0.21	0	0.25	0
Dugway Gate	0	0	0	0.17	0	0	0.05	0	0	0.04	0	0
Dugway Camp	0	0	0	0.13	0	0	0.17	0	0	0.01	0	0

[a] "nd": no data. The R_f values indicate the range within which the spots occur. Based on the clarity and inhibition of the spot, we scored the strength of the spot 1.00 for strong, 0.25 for weak and 0.025 for very weak. The antifungal value for a specific spot from a population was calculated by averaging the values among all six replicates. The highest antifungal value that a spot could attain was 1.0, which meant that the spot was strong among all six replicates.

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EFFECT OF PARTHENIN—A SESQUITERPENE LACTONE FROM *Parthenium hysterophorus*—ON EARLY GROWTH AND PHYSIOLOGY OF *Ageratum conyzoides*

HARMINDER P. SINGH,[1,*] DAIZY R. BATISH,[1] R. K. KOHLI,[1] DINESH B. SAXENA,[2] and VANDANA ARORA[1]

[1]*Department of Botany*
Panjab University
Chandigarh 160 014, India

[2]*Division of Agricultural Chemicals, Shashtri Bhavan*
Indian Agricultural Research Institute, PUSA
New Delhi 110 012, India

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Abstract—A study was undertaken to assess the effect of parthenin—a sesquiterpene lactone from *Parthenium hysterophorus*—on the germination, growth, and some associated physiological changes in *Ageratum conyzoides*. The study reveals that germination and growth of *A. conyzoides* was severely reduced by parthenin in the concentration range of 50, 100, and 200 μM, while at 400 μM a complete inhibition of germination was observed. The radicle length of *A. conyzoides* was reduced more than the plumule length. Further, the content of the photosynthetic pigment chlorophyll was appreciably reduced in the leaves of test plants spray treated with parthenin at 200 μM concentration. However, this inhibitory effect declined with the passage of time and at 10 days after treatment only a 25% reduction in chlorophyll content was observed compared to 76% on the first day after spray. Likewise, the cellular respiration measured through 2,3,5-triphenyl tetrazolium chloride was found to be less in the treated leaves, and the inhibitory response also declined with time. The content of proteins and carbohydrates decreased with the passage of time after parthenin treatment. The specific activities of the enzymes protease and α- and β-amylase were also adversely affected in the parthenin treated leaves. In the case of protease, the activity decreased with passage of time while those of the amylases increased. Thus, we conclude that parthenin affects the germination and growth of *A. conyzoides* by altering the contents of some macromolecules

* To whom correspondence should be addressed. E-mail: hpsingh_01@yahoo.com

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and the specific activities of some enzymes. Such observations may be helpful in further exploring parthenin's mode of action.

Key Words—Parthenin, allelochemical, growth, chlorophyll content, enzyme activities, protein content, carbohydrate content.

INTRODUCTION

Plants produce many compounds that play a useful function in their interaction with the environment (Paiva, 2000). These compounds often make plants more successful in terms of competition and reproductive vigor. They are also often responsible for imparting allelopathic activity to plants, including microbes (Rice, 1984). One of the applied aspects of allelopathy is to use allelochemicals as natural herbicides for weed management. Although natural product allelochemicals have been commercially utilized as insecticides, fungicides, and bactericides, their role as natural herbicides has been neglected. One significant aspect of their use as herbicides is that they are relatively environmentally safe and possess novel target sites different from those of traditional herbicides and can, thus, be useful in controlling weeds, particularly those resistant to herbicides (Devine et al., 1993; Dayan et al., 1999b; Duke et al., 2000; Macias et al., 2001).

Sesquiterpene lactones constitute a large group of compounds exhibiting diverse biological activity (Rodriguez et al., 1976; Picman, 1986; Fischer et al., 1989; Macias et al., 1996). These are active even at low concentrations and exhibit specific structure–activity relationships (Beekman et al., 1997; Macias et al., 1999, 2001). Among several biologically active compounds, the phytotoxic potential of sesquiterpene lactones is widely known (Dalvi et al., 1971; Del Amo and Anaya, 1978; Saxena et al., 1991; Macias et al., 1993, 1996, 1998; Dayan et al., 1999a; Galindo et al., 1999). Parthenin, a sesquiterpene lactone and major constituent of tropical weed *Parthenium hysterophorus*, is a known inhibitor of growth (Kanchan and Jayachandra, 1980; Saxena et al., 1991; Kohli et al., 1993; Batish et al., 1997). It is present in the aerial parts of the weed and is known to have a number of properties, such as allerginicity, allelopathic potential, autotoxicity, and toxicity to livestock (Kohli and Rani, 1994). In addition, recently, its pesticidal potential has been explored in terms of ovicidal, antifeedant, herbicidal, and nematicidal activity (Datta and Saxena, 2001). However, little has been done to explore its herbicidal potential except for a toxicity study in aquatic weeds (Pandey, 1996) and one in terrestrial weeds (Batish et al., 1997). For a compound to be an ideal herbicide, it should inhibit seed germination of target species completely with little or no effect on nontarget plants such as crops. Species specificity, thus, is an important aspect of natural herbicides. Parthenin exhibited this property in experiments with *Triticum aestivum* (wheat) and *Ageratum conyzoides* (bill goat weed) where it inhibited germination of *A. conyzoides* but not wheat (Batish et al., 1997).

However, there are no further reports on the phytotoxicity of parthenin towards *A. conyzoides*—a weed commonly associated with wheat in hilly tracts of north India. The present study was conducted to determine the effect of parthenin on the germination and seedling growth of *A. conyzoides* and on related physiological and biochemical characteristics such as chlorophyll content, respiratory activity, water content, soluble protein and carbohydrate concentrations, and protease, α-amylase, and β-amylase enzyme activities.

METHODS AND MATERIALS

Extraction of Parthenin. Parthenin was extracted from the shade-dried powdered leaves of *Parthenium hysterophorus* L. (collected from locally growing wild stands) following the method of Saxena et al. (1991). Parthenin, crystallized as fine colorless needles, was characterized with IR, PMR, and MS (Saxena et al., 1991). Its melting point was 165°C and purity was >98%. For growth experiments, a stock solution of 400 μM parthenin was prepared by dissolving the requisite amount in 2 ml absolute alcohol and then bringing the final volume to 1 l with distilled water. The stock solution was diluted to get solutions of 200, 100, and 50 μM concentrations. The concentration of alcohol in the final solution was 0.2%. The same amount of alcohol added to distilled water served as control.

Growth Experiments. Healthy seeds of *Ageratum conyzoides* L. (bill goat weed; family Asteraceae) were collected from the locally growing wild stands. The effect of different concentrations of parthenin was studied on the germination and early growth of *A. conyzoides* in a laboratory bioassay. Fifty seeds were placed for imbibition in different concentrations of parthenin solutions or distilled water containing 0.2% alcohol (a control) and allowed to imbibe for 18 hr. Imbibed seeds were placed equidistantly on Whatman No. 1 filter paper moistened with 7 ml of respective treatment solution in a 15-cm-diam. Petri dish. For each treatment, five replicates were maintained. All Petri dishes were placed in a seed germinator maintained at 23 ± 2°C, 75 ± 2% relative humidity, 16L:8D photoperiod, and 270 μW/cm^2 energy. After 7 d, the number of seeds germinated, radicle and plumule lengths of the germinated seeds, and their dry weights were measured. The entire experiment was repeated twice. Based on the growth experiment, a parthenin concentration of 200 μM was selected for further studies.

Treatment of Plants. Plants of *A. conyzoides* were raised from locally collected seeds in 10-cm-diam. pots in a growth chamber at 25 ± 2°C temperature, 75 ± 2% relative humidity, 16L:8D photoperiod, and photon flux density of approximately 150 μmol photons/m^2/sec. For this experiment, 1200 g of garden soil (soil–sand, 3:1, w/w) were placed in each pot, and five seeds of *A. conyzoides* were

sown. A week after emergence, these were thinned to two plants per pot. When the plants were 4 wk old, they were sprayed with 200 μM solution of parthenin (or distilled water with 0.2% alcohol as control) so that each plant received 10 ml of treatment solution. The treatment was given for three consecutive days, and for each treatment there were 10 replicates. On the day after the last treatment (hereafter referred to as the first day), the leaves were plucked and used to estimate chlorophyll content, respiratory activity, water-soluble protein and carbohydrate content, and enzyme activities. Similarly, the leaves were again plucked from the treated plants on days 5 and 10 for the estimations.

Chlorophyll Content. Chlorophyll from 30 mg of parthenin-treated or control-treated leaves was extracted in 4 ml of dimethyl sulfoxide (DMSO) as per the method of Hiscox and Israelstam (1979). It was quantified spectrophotometrically using the equation of Arnon (1949) and expressed on dry weight basis as suggested by Daizy and Kohli (1991).

Cellular Respiration. Respiratory activity was determined indirectly using 2,3,5-triphenyl tetrazolium chloride following the method of Steponkus and Lanphear (1967) in which the red formazan formed traps the oxygen released through the respiratory chain, thus allowing the indirect measurement of respiration. It was calculated using the following formula and expressed with respect to control.

$$\%\ \text{Respiratory activity} = \frac{\text{Absorbance of parthenin treated samples at 530 nm}}{\text{Absorbance of control samples at 530 nm}} \times 100$$

Determination of Water-Soluble Proteins and Carbohydrates. For estimating water-soluble proteins and carbohydrates, parthenin treated leaf tissue was crushed in acetone and petroleum ether to remove water, phenol, and lipids. The dried powder was used for the estimation of protein and carbohydrate content. Protein content was determined as per Lowry et al. (1951). The carbohydrate content was estimated using anthrone reagent as per Loewus (1952).

Assay of Enzyme Activities. For preparing crude enzyme extracts, parthenin-treated or control leaf tissue was homogenized in a prechilled mortar and pestle using a small amount of acid-washed sand (pH 7) and 5 ml of 0.1 M phosphate buffer (pH 7). Homogenates were centrifuged at 8000 g, 17,000 g, and 27,000 g for 7, 11, and 5 min, respectively, at a rotor temperature of 4°C in an IEC ultracentrifuge. The supernatant fraction was assayed for enzyme activity. Specific activity of protease was assayed using casein (1% in 0.1 M phosphate buffer, pH 6.0) as a substrate as per Basha and Beevers (1975). Specific activity of α-amylase was assayed as per Muentz (1977) using starch as a substrate solution. The activity of β-amylase was measured following the method of Bernfeld (1951) modified by Dure (1960).

Statistical Analysis. Percent changes in germination, radicle and plumule length, chlorophyll content, cellular respiration, protein and carbohydrate content, and enzyme activities were analyzed using a two-sample t test at α levels of 0.05 and 0.01.

RESULTS AND DISCUSSION

Within the 50–200 μM range of parthenin treatment concentrations, the germination of *A. conyzoides* was significantly reduced. At 400 μM concentration, it was completely inhibited (Table 1). Not only the germination, but also the further growth of the seedlings in terms of radicle and plumule length and seedling dry weight was significantly reduced, particularly at the parthenin concentrations of 100 and 200 μM. A relatively greater effect was observed on the radicle than plumule. At the 200 μM concentration of parthenin, radicle growth of *A. conyzoides* was reduced by nearly 88%. The pattern of growth inhibition was of a dose–response type, i.e., inhibition increased with increasing concentrations. Although the reasons for inhibition/reduction of growth of *A. conyzoides* could not be determined from the present study, recent studies have revealed that allelochemicals such as quassinoids, artemisinin, and volatile cineoles inhibit mitosis and, thus, growth of plants (Dayan et al., 1999a,c; Romagni et al., 2000). Parthenin may cause inhibition of *A. conyzoides* in a similar manner.

Spray treatment with 200 μM parthenin solution on *A. conyzoides* plants grown in pots, showed the chlorophyll content to be significantly reduced on days 1, 5, and 10 after the treatment (Table 2). The chlorophyll content was $<70\%$ of the control on days 1 and 5. However, by day 10 chlorophyll content showed only a 25% reduction relative to the control (Table 2). The reduction in chlorophyll has a direct bearing on photosynthesis. In fact, many allelochemicals are known to reduce chlorophyll content in target species (Einhellig and Rasmussen, 1979; Daizy and Kohli, 1991; Baziramakenga et al., 1994; Dayan et al., 1999a; Romagni et al.,

TABLE 1. EFFECT OF PARTHENIN ON GERMINATION AND EARLY GROWTH OF *A. conyzoides*[a]

Concentration (μM)	Germination (%)	Plumule length (cm)	Radicle length (cm)	Dry weight (mg/seedling)
0 (Control)	90.0 ± 2.5	3.7 ± 0.31	0.97 ± 0.04	1.90 ± 0.36
50	$72.4 \pm 2.8^{*}$	$2.6 \pm 0.42^{*}$	$0.72 \pm 0.12^{*}$	$1.14 \pm 0.21^{*}$
100	$59.4 \pm 1.5^{**}$	$1.9 \pm 0.24^{**}$	$0.57 \pm 0.08^{*}$	$0.76 \pm 0.17^{*}$
200	$10.4 \pm 0.5^{**}$	$1.1 \pm 0.20^{**}$	$0.12 \pm 0.05^{**}$	$0.30 \pm 0.09^{**}$
400	-	-	-	-

[a] * and ** mean significant at $P < 0.05$ and 0.01, respectively.

TABLE 2. TOTAL CHLOROPHYLL CONTENT AND RESPIRATORY ACTIVITY IN LEAVES OF ONE MONTH-OLD PLANTS OF *A. conyzoides* SPRAY TREATED WITH 200 μM OF PARTHENIN[a]

Days after treatment	Total chlorophyll (mg/g tissue)		Respiratory activity (% of control)
	Control	Treated	
1	5.6 ± 0.31	$1.4 \pm 0.25^{**}$ (75.0)	$53.4 \pm 1.50^{**}$ (46.6)
5	5.7 ± 0.35	$1.6 \pm 0.14^{**}$ (71.9)	$68.1 \pm 2.70^{*}$ (31.9)
10	5.8 ± 0.27	$4.4 \pm 0.62^{*}$ (24.1)	$78.3 \pm 1.40^{*}$ (21.7)

[a] * and ** mean value is significant from respective control at $P < 0.05$ and 0.01, respectively. Numbers in parenthesis represent percent decrease from respective control.

2000; Singh et al., 2002), but it is unknown whether loss of chlorophyll results from the inhibition of chlorophyll biosynthetic pathway or its degradation. Respiration in the parthenin-treated plants was also reduced (Table 2), with the reduction greatest on the first day (47%), and diminishing with time (22% on day 10) (Table 2). Such an inhibitory effect of parthenin on the respiration of germinating mung bean has also been reported by Kohli et al. (1993). The effect of parthenin on respiration may affect cell metabolism, leading to impaired growth of target species. However, nothing is known about the mechanism of action. Picman (1986) pointed out that parthenin, like other sesquiterpenes, may bind to sulfhydryl bonds of amino acids and proteins and cause irreversible alkylation of key enzymes, thereby impairing general metabolism and causing growth inhibition. In order to establish such a mechanism, we studied the amount of total protein content and its hydrolyzing enzyme protease in the parthenin-treated plants. Furthermore, because parthenin affected the respiration in the target plants and because carbohydrates act as major substrates for this process, we also estimated the amount of carbohydrates and the specific activity of their hydrolyzing enzymes—amylases. Such types of biochemical estimations provide clues regarding the mode and mechanism of action (Dayan et al., 2000).

Treatment with parthenin adversely affected the amounts of some macromolecules, such as water-soluble proteins and carbohydrates, and the specific activities of the enzymes, *viz.*, protease and α- and β-amylase. The protein content decreased in the parthenin-treated plants on the first day, and with the passage of time the decrease was even more pronounced i.e., it was nearly 80% on the fifth and tenth days after the parthenin treatment compared to about 40% on the first day (Table 3). The trend of changes in the carbohydrate content was also similar to that observed for the proteins (Table 3). The specific activity of protease—protein hydrolyzing enzyme—was also reduced in the parthenin treated *A. conyzoides* plants. This indicates an overall affect on protein metabolism. However, unlike protease,

TABLE 3. EFFECT OF PARTHENIN TREATMENT ON AMOUNT OF WATER-SOLUBLE PROTEINS AND CARBOHYDRATES IN LEAVES OF ONE-MONTH-OLD PLANTS OF *A. conyzoides*[a]

Days after treatment	Protein content (mg/g tissue)		Carbohydrate content (mg/g tissue)	
	Control	Treated	Control	Treated
1	32.7 ± 2.40	$19.9 \pm 1.30^{*}$ (39.1)	28.4 ± 1.27	$18.7 \pm 1.19^{*}$ (34.2)
5	32.5 ± 1.90	$6.82 \pm 1.10^{**}$ (79.0)	27.6 ± 2.31	$10.8 \pm 1.37^{**}$ (60.9)
10	31.6 ± 1.70	$6.02 \pm 1.50^{**}$ (80.9)	29.2 ± 2.48	$7.40 \pm 1.24^{**}$ (74.7)

[a] * and ** mean significant from respective control at $P < 0.05$ and 0.01, respectively. Figures in parenthesis represent percent decrease from respective control.

the specific activity of carbohydrate hydrolyzing enzymes—α- and β-amylase—increased with parthenin treatment compared to respective controls (Table 4).

It is clear that parthenin exerts an overall inhibitory effect on the growth and physiology of *A. conyzoides*. The adverse effect on protein and carbohydrate metabolism, as indicated by significant alterations in their amounts, does not explain anything regarding parthenin's mode of action; however, the results serve as indicators for further studies on the mode of action. Pandey (1996) reported that parthenin causes several physiological changes in the water hyacinth, such as damage to cellular membranes, loss of dehydrogenase activity in the roots, and loss of chlorophyll in the leaves, resulting in growth inhibition. Earlier, Pandey et al. (1993) pointed out that inhibitors of *P. hysterophorus* may be acting through their effects on various macromolecules. The present study supports this. Another sesquiterpene lactone, dehydrozaluzanin-C, present in many composites, is phytotoxic to several plant species and causes rapid and pronounced cellular leakage, resulting in secondary effects, such as loss of membrane integrity and affecting amino acid biosynthetic pathways (Galindo et al., 1999; Macias et al., 2000).

Under natural conditions, parthenin released from different parts of the weed may accumulate in toxic concentrations in the soil environment, thereby affecting the population dynamics of associated vegetation and bringing about shifts in weed flora. Additionally, parthenin may also affect other ecological interactions such as antimicrobial, antifungal, and nematicidal activities, thereby providing defense against a number of organisms (Kohli and Rani, 1994).

Parthenin possesses an α-methylene-γ-lactone and an α,β-unsubstituted cyclopentenone ring, around a seven-membered ring, which makes it chemically reactive towards a wide variety of biological nucleophiles. The presence of the unsubstituted ring provides potential for chemical modification. Several derivatives of parthenin have already been prepared, and many of these exhibit greater activity than parthenin itself (Saxena et al., 1991; Datta and Saxena, 2001). Vaughan and

TABLE 4. SPECIFIC ACTIVITIES OF PROTEASE, AND α- AND β-AMYLASE IN LEAVES OF ONE-MONTH-OLD PLANTS OF *A. conyzoides* AFTER SPRAY TREATMENT WITH 200 μM PARTHENIN[a]

Days after treatment	Protease activity (μg/hr/mg protein)		α-Amylase (μg/min/mg protein)		β-Amylase (μg/min/mg protein)	
	Control	Treated	Control	Treated	Control	Treated
1	17.5 ± 0.65	$9.30 \pm 0.85^{*}$ (−46.9)	0.061 ± 0.002	$0.081 \pm 0.003^{*}$ (+32.8)	8.96 ± 0.70	$21.9 \pm 0.49^{**}$ (+144.4)
5	17.3 ± 0.42	$5.92 \pm 0.97^{**}$ (−65.8)	0.067 ± 0.001	$0.091 \pm 0.001^{*}$ (+35.8)	9.17 ± 0.83	$19.3 \pm 0.93^{**}$ (+110.4)
10	17.1 ± 0.32	$4.01 \pm 0.71^{**}$ (−76.6)	0.074 ± 0.004	$0.109 \pm 0.005^{**}$ (+47.3)	9.00 ± 1.14	$18.1 \pm 1.24^{**}$ (+101.1)

[a] * and ** mean value is significant from respective control at $P < 0.05$ and 0.01, respectively. Numbers in parenthesis represent percent change from respective control; "−" indicates decrease; "+" indicates increase.

Spencer (1993) indicated that important characteristics for any natural compound chosen to be tested for herbicidal activity are the presence of reactive sites in the structures for chemical alterations and species specificity. Parthenin has potential sites for attachment of other functional groups and has demonstrated species specificity, affecting germination of *Ageratum conyzoides* with little or no effect on wheat (Batish et al., 1997). Thus, it could be an ideal molecule for development as a natural herbicide. Preliminary comparisons with known herbicides suggest that its phytotoxic activity is comparable to 2,4-D (data not shown). The dissipation of the parthenin from the environment in about 30 days (Pandey, 1996) may be of ecological significance in its use as a novel agrochemical compared to synthetic herbicides.

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(*Z*)-8-HEPTADECENE FROM INFESTED CELLS REDUCES THE REPRODUCTION OF *Varroa destructor* UNDER LABORATORY CONDITIONS

FRANCESCO NAZZI,* NORBERTO MILANI, and GIORGIO DELLA VEDOVA

Dipartimento di Biologia Applicata alla Difesa delle Piante
Università di Udine
Udine, Italy

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Abstract—The parasitic mite *Varroa destructor* Anderson and Trueman, the most serious threat to apiculture in many countries of the world, reproduces inside honeybee brood cells. Previous research, using artificial cells for rearing the mite on an *Apis mellifera* larva, indicated that semiochemicals affecting the reproduction of *Varroa destructor* are released into such cells. In order to isolate these semiochemicals, infested artificial cells were extracted with hexane and the extract fractionated twice. Several unsaturated hydrocarbons were identified in the active fraction; some of them were released in higher amounts in case of infestation and were, therefore, bioassayed for their effect on the mite's reproduction. Of five alkenes tested under laboratory conditions, (*Z*)-8-heptadecene, caused a 30% reduction in the mean number of offspring of mites reared in cells treated with this compound.

Key Words—Alkenes, *Apis mellifera*, honeybee, hydrocarbons, reproduction, semiochemical, *Varroa destructor*, (*Z*)-8-heptadecene.

INTRODUCTION

The mite *Varroa destructor* Anderson and Trueman (Mesostigmata Varroidae), which was recently discriminated from *V. jacobsoni* Oudemans (Anderson and Trueman, 2000), is the major pest of the western honeybee (*Apis mellifera*). Unless effective treatments are carried out, bee colonies are destroyed by the mite (Sammataro et al., 2000). Effective control of the parasite has become more difficult since the appearance and spread of mite populations that are resistant to

* To whom correspondence should be addressed. E-mail: francesco.nazzi@pldef.uniud.it

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widely used acaricides (Milani, 1999). The need for alternative control methods suggested research on the honeybee–varroa relationship to find out what enables some bee strains to tolerate the mite (Rosenkranz, 1999). Reduced reproduction of the mite is regarded as the most important factor of tolerance (Fries et al., 1994).

Varroa reproduces on immature stages of the honeybee inside sealed brood cells. In multiple infested cells, a density-dependent reduction of varroa reproduction was observed under both natural (Piletskaya, 1982; Moosbeckhofer et al., 1988; Fuchs and Langenbach, 1989; Martin, 1995) and laboratory conditions (Nazzi and Milani, 1996). Laboratory studies using gelatin cells for rearing the mite on its natural host showed that inhibition of reproduction is caused by substances that are released into infested cells and can be extracted with suitable solvents (Nazzi and Milani, 1996). The aim of this research was to identify such factors.

METHODS AND MATERIALS

Biological Material. Bee larvae and *V. destructor* mites used in all the experiments were taken from *A. mellifera* colonies maintained in Udine (northeastern Italy). The mites and bee larvae were obtained from brood cells capped 0–15 hours before (0–15 pc), according to Chiesa et al. (1989).

Bioassay. A solution of the stimulus to be tested in 10 μl of hexane was applied to the interior of 6.5 mm ID gelatin cells (Capsugel, Coni-Snap, size 1), then one varroa mite and a *0–15 PC* bee larva were inserted into each cell. Gelatin cells treated with 10 μl of hexane were prepared as above and used as a control. Cells were mantained at $34.5 \pm 0.5^{\circ}$C, 75% relative humidity in an incubator for the duration of the experiment. After 10 d, the cells were opened and inspected under a dissecting microscope; the presence and the number of offspring per mite in each cell was noted. Only cells with a normal bee pupa were considered.

Extraction of Inhibiting Factors. Infested cells, in which a bee larva infested with a mite had developed, were obtained according to Nazzi and Milani (1996); only cells with a normal bee pupa and a varroa mite that had reproduced were used. At the end of the rearing cycle, the bee pupae, the varroa mites, and their offspring were removed, and the cells were filled with 380 μl of hexane. After 15 min, the solvent from all extracted cells was transferred into a vial and stored at -20°C.

Fractionation of the Active Extract. The extract of a convenient number of infested cells (normally 30–100 cells) was concentrated under nitrogen and fractionated on a column obtained by packing a Pasteur pipet with 300 mg of silica gel (200–400 mesh, 60Å). The column was eluted with 3 ml of hexane (F1), 10% ether–hexane (F2), and ether (F3). The fractions were then concentrated under nitrogen to 1 cell equivalent (1 CE) per 10 μl of hexane and bioassayed. The experiment was replicated 4 times; about 65 cells per group gave useful information that was used for the statistical analysis.

Saturated and unsaturated hydrocarbons contained in the apolar fraction resulting from the first fractionation (F1) were separated using a column packed with 300 mg of 10% silver nitrate on silica gel (200–400 mesh, 60Å). The column was eluted with 3 ml of hexane (F1A) and ether (F1B). The ether fraction was reduced under nitrogen and passed through a silica column that was eluted with hexane to remove silver ions. The fractions were then concentrated under nitrogen to 1 CE/10 μl hexane and bioassayed. The experiment was replicated 6 times; about 85 cells per group gave useful results that were used for the statistical analysis.

Chemical Identification. Ten infested cells and ten uninfested cells were extracted with hexane, the extracts were pooled and analyzed with a Varian 3400 gas chromatograph coupled to a Varian Saturn 2000 mass spectrometer. The column (30 m $\times$ 0.25 mm ID, CP-SIL 8) was maintained at 40°C for 1 min, then programmed at 10°C/min to 320°C. The carrier gas was helium. The comparison of the percentage composition of the two extracts is based on three replicates of the analysis.

Alkenes released in infested and uninfested cells were also studied by means of solid phase microextraction (SPME). Infested and uninfested gelatin cells were prepared as described above and held in an incubator at 34.5 ± 0.5°C and 75% relative humidity. After 24 hr, five cells of each type were sealed in an air-tight vial, then a SPME fiber (100 μm polydimethylsiloxane) was exposed to the volatiles emitted in the vial for 1 hr and analyzed. Chromatographic conditions were as above, except that the column upper temperature was 250°C.

The compounds in the extracts were identified by comparison of the spectra and the retention times with those of authentic standards that had been either purchased or synthesized. The double-bond position of unsaturated hydrocarbons detected in crude extracts was assessed by derivatization with dimethyl disulfide (Carlson et al., 1989). To determine the stereochemistry of alkenes, 8 CE of the extract were co-chromatographed with authentic standards on silver nitrate impregnated TLC plates. The sections of the plate corresponding to the *Z* and *E* alkenes were extracted with ether and analyzed by GC-MS.

(*Z*)-9-Heneicosene (9C21:1) and (*Z*)-9-tricosene (9C23:1) were purchased from Sigma-Aldrich. (*Z*)-6-Pentadecene (6C15:1), (*Z*)-6-heptadecene (6C17:1), (*Z*)-7-heptadecene (7C17:1), (*Z*)-8-heptadecene (8C17:1), (*Z*)-9-nonadecene (9C19:1), which were identified in the active extract but were not commercially available, were synthesized by partial hydrogenation of the corresponding alkyne using Lindlar's catalyst. Alkynes were obtained by alkylation of a convenient terminal alkyne with 1-bromoalkanes of suitable chain length (Sonnet, 1984).

Bioassay of Compounds Identified in Active Fraction. The alkenes 6C15:1, 8C17:1, 9C19:1, 9C21:1 and 9C23:1 were bioassayed at a concentration of 100 ng/cell. The experiment was replicated 4 times. The number of cells used in each case is given in Table 4. Mortality of mites in cells treated with each compound was compared to that in control cells.

Effect of Double-Bond Position on Biological Activity of Heptadecene. This was assessed by comparing the activity of three different isomers of heptadecene [(*Z*)-6-heptadecene, (*Z*)-7-heptadecene, and (*Z*)-8-heptadecene] on the reproduction of varroa mites reared in cells treated with those compounds. The concentration of chemicals was 100 ng/cell. The experiment was replicated 3 times; about 50 cells per group gave useful information that was used for the statistical analysis.

Statistical Analysis of Data. The offspring per varroa in treated and control cells were compared by means of a two-sample randomization test (Manly, 1997); the randomization distribution was resampled 10^6 times with a computer program written for this purpose. The varroa mortality inside treated cells was compared to that in control cells by means of the *G* test (Sokal and Rohlf, 1995).

RESULTS

Fractionation of the Active Extract. The reproduction of the mites reared in cells treated with the apolar fraction (F1) containing the hydrocarbons was significantly lower than that observed in cells treated with the solvent alone. The medium polarity fraction (F2) showed some activity as well (Table 1), but, due to the poor efficiency of the column used here, this was most likely due to hydrocarbons that had not yet been eluted from the column rather than to other compounds of higher polarity as confirmed by GC analysis of the fraction. Several odd chain length alkanes, alkenes, and branched alkanes were identified in the apolar fraction (F1). Only fraction F1B from the second fractionation, which contained the alkenes, reduced the reproduction of the mites reared in treated cells (Table 2).

Chemical Identification. The monoenes listed in Table 3 were identified in the active fraction; the double bond was located nine carbon atoms from the end of

TABLE 1. OFFSPRING PER MITE IN CELLS TREATED WITH SOLVENT (CONTROL) OR FRACTIONS (F1, F2, F3) OF EXTRACT OF REARING CELLS CONTAINING A BEE LARVA INFESTED BY ONE VARROA MITE[a]

Replication	Control	F1	F2	F3
1	1.5 (10)	1.0 (11)	0.7 (20)	1.8 (16)
2	1.4 (11)	1.2 (15)	2.4 (13)	1.4 (9)
3	2.1 (18)	1.5 (14)	1.5 (11)	2.3 (16)
4	2.0 (26)	0.9 (28)	1.0 (26)	2.3 (22)
Total	1.9 (65)	1.1 (68)	1.3 (70)	2.0 (63)
P		0.004	0.017	0.305

[a] The number of cells considered for each experimental group is given in parentheses.
P represents the significance of the differences beetween each of the fractions and the control.

TABLE 2. OFFSPRING PER MITE IN CELLS TREATED WITH SOLVENT (CONTROL) OR FRACTIONS (F1A AND F1B) OBTAINED FROM SECOND FRACTIONATION[a]

Replication	Control	F1A	F1B
1	2.1 (24)	2.7 (16)	1.6 (14)
2	1.1 (20)	1.5 (13)	1.7 (20)
3	2.9 (16)	2.0 (21)	1.9 (22)
4	3.8 (5)	2.0 (8)	1.9 (9)
5	2.1 (11)	2.1 (8)	1.4 (8)
6	2.2 (14)	1.4 (14)	1.2 (15)
Total	2.1 (90)	2.0 (80)	1.6 (88)
P		0.306	0.029

[a] The number of cells considered for each experimental group is given in parentheses.
P represents the significance of the differences beetween each of the fractions and the control.

the chain for most alkenes. Although the presence of small quantities of *E* isomers of the alkenes found in the extracts can not be ruled out, the configuration of the unsaturated hydrocarbons was *Z* in accordance with previous studies on cuticular hydrocarbons of the honeybee (Blomquist et al., 1980).

The amount of alkenes containing 17–25 carbon atoms was higher in the active extract of infested cells than in the inactive extract of uninfested cells (Figure 1). The absolute quantity of alkenes per cell ranged from nanograms, in the case of shorter alkenes, to hundreds of nanograms in the case of longer ones.

SPME analyses showed that higher quantities of heptadecene and pentadecene are emitted in infested cells. On the other hand, traces of alkadienes were also found

TABLE 3. MONOENES IDENTIFIED IN EXTRACT OF INFESTED CELLS[a]

Compound name	Formula	Abundance (%)*	Principal isomers	Other isomers
Pentadecene	$C_{15}H_{30}$	traces	?	
Heptadecene	$C_{17}H_{34}$	0.15	8	
Nonadecene	$C_{19}H_{38}$	7	9	7
Heneicosene	$C_{21}H_{42}$	1	9	7, 8, 10
Tricosene	$C_{23}H_{46}$	12	9	7, 8, 10, 11
Pentacosene	$C_{25}H_{50}$	19	9	7, 8, 10
Heptacosene	$C_{27}H_{54}$	13	9	7, 8, 10
Nonacosene	$C_{29}H_{58}$	8	9	7, 8, 10
Hentriacontene	$C_{31}H_{62}$	15	8, 9, 10	
Tritriacontene	$C_{33}H_{66}$	30	10	8, 9, 11, 12

[a] The abundance of each alkene is expressed as the percentage of the total amount of hydrocarbons. Isomers accounting for more than 25% are regarded as principal isomers, the other isomers account for more than 1%.

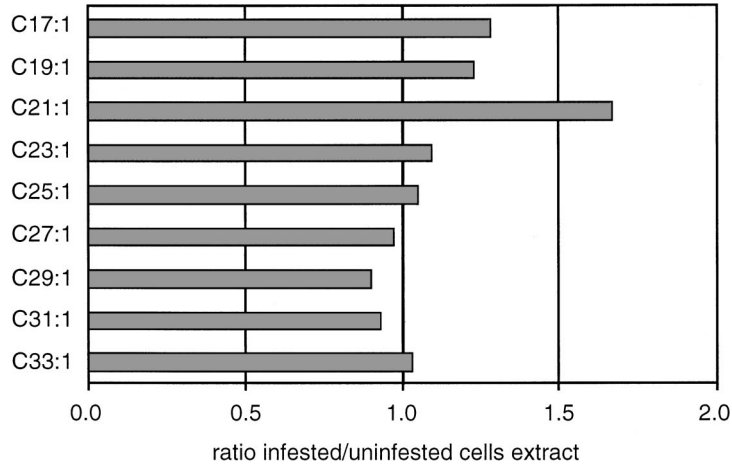


FIG. 1. Comparison of alkenes extracted from rearing cells containing a bee larva infested by one varroa mite or uninfested. The ratio between the amount recovered in infested and uninfested cells is given.

in the active fraction, but, due to their scarcity, no further studies were possible on these compounds.

Bioassay of Compounds Identified in Active Fraction. Treatment of cells with (*Z*)-8-heptadecene significantly reduced the offspring per female mite, while no significant differences from the control were found for cells treated with (*Z*)-7-pentadecene, (*Z*)-9-nonadecene, (*Z*)-9-heneicosene, and (*Z*)-9-tricosene (Table 4). No significant effect on mite mortality was caused by (*Z*)-8-heptadecene (mite mortality in control cells: 9%, mite mortality in treated cells: 10%; $P > 0.8$).

TABLE 4. OFFSPRING PER MITE IN CELLS TREATED WITH SOLVENT (CONTROL) OR 100 ng OF DIFFERENT ALKENES (TREATED)[a]

Compound	Control	Treated	*P*
6C15:1	2.1 (63)	2.6 (60)	n.s.
8C17:1	2.4 (77)	1.7 (80)	0.005
9C19:1	2.2 (54)	2.2 (54)	n.s.
9C21:1	2.1 (33)	3.0 (67)	n.s.
9C23:1	2.2 (57)	2.3 (74)	n.s.

[a] The number of cells considered for each experimental group is given in parentheses.
Each row represents the total of four replicates.
P represents the significance of the difference beetween control and treatment.

TABLE 5. OFFSPRING PER MITE IN CELLS TREATED WITH SOLVENT (CONTROL) OR 100 ng OF THREE ISOMERS OF HEPTADECENE[a]

Replication	Control	6C17:1	7C17:1	8C17:1
1	2.6 (14)	2.4 (19)	2.5 (22)	2.0 (23)
2	2.3 (16)	2.3 (22)	2.8 (17)	1.5 (14)
3	3.2 (11)	1.7 (17)	2.2 (13)	2.0 (14)
Total	2.6 (41)	2.1 (58)	2.5 (52)	1.9 (51)
P		0.108	0.372	0.028

[a] The number of cells considered for each experimental group is given in parentheses.
P Represents the significance of the differences beetween each isomer and the control.

Effect of Double-Bond Position on Biological Activity of Heptadecene. (*Z*)-8-Heptadecene was active in inhibiting the reproduction of the varroa mite at the concentration tested, while the other two isomers tested did not cause any significant decrease in reproduction (Table 5).

DISCUSSION

The cuticular hydrocarbons of the honeybee and the varroa mite have been studied by several researchers (Blomquist et al., 1980; Francis et al., 1985; Carlson et al., 1989; Nation et al., 1992; Rickli et al., 1994). More recently Salvy et al. (2001) studied the hydrocarbon profile of infested and uninfested bee larvae, pupae, and emerging bees. However, the presence of unsaturated hydrocarbons shorter than 21 carbon atoms has not been reported so far. By extracting artificial rearing cells, we identified three additional alkenes: pentadecene, (*Z*)-8-heptadecene, and (*Z*)-9-nonadecene. (*Z*)-8-Heptadecene, which is released in higher quantities in case of infestation, caused a significant reduction in the reproduction of the mite without causing increased mortality; this rules out a toxic effect of the compound at the concentration tested.

The analysis of cuticular hydrocarbons of uninfested bee larvae and the varroa mite revealed the presence of traces of heptadecene in both cases. An effective technique for rearing *V. destructor* on an artificial diet has not yet been developed. Thus, it is not possible to ascertain directly whether heptadecene found in infested cells is produced by the bee larva following parasitization or by the varroa mite itself.

Mechanical damage produced by the mite feeding on its host can be simulated by piercing the cuticle of the bee larva with a fine pin; in this way, one can induce stress in the larva somewhat similar to that induced by the mite but without the mite being present. By rearing the mite on its host in artificial cells that had

been previously used for rearing a bee larva injured in such a way, we observed a reduction in the reproduction of the parasite in comparison to that observed in cells that had been previously occupied by healthy larvae (offspring per mite in cells that contained a wounded larva in the previous rearing cycle: 1.33, offspring per mite in cells that contained a healthy larva in the previous rearing cycle: 2.35; $P = 0.06$). This suggests that compounds affecting mite reproduction could be produced by a bee larva under stress regardless of the presence of the mite. Actually, evidence that environmental stress can alter the hydrocarbon composition of the insect cuticle has already been reported (Howard et al., 1995 and citations therein). Therefore, it is likely that (*Z*)-8-heptadecene represents an allomone released by the host under stress that affects the reproduction of its parasite.

(*Z*)-8-Heptadecene has already been described as a semiochemical for the mite *Acarus immobilis* (Sato et al., 1993) and was recovered among the volatiles produced by *Dermatophagoides pteronyssus* (Kuwahara et al., 1990). However, to our knowledge, no effect of this compound on the reproduction of any mite or tick has been noted so far. An unidentified fecundity-reducing semiochemical was reported for *Argas arboreus*, but it appeared to be a pheromone produced under crowded conditions by the tick itself (Khalil, 1984).

Although no data exist yet on the correlation between infestation level and heptadecene production by the larvae, the effect of heptadecene on varroa reproduction is similar to that observed in multiple infested artificial cells (Nazzi and Milani, 1996). The identification of this inhibitor of reproduction in *V. destructor* could help elucidate the mechanism of the density-dependent reduction in mite reproduction observed in multiple infested cells.

The identified semiochemicals could be exploited for the control of the parasite. Due to the high number of generations per year in *V. destructor*, even a small reduction in the reproductive rate could have a significant impact on the development of infestation. Identification of such a chemical could make it possible to better understand the factors conferring tolerance to the varroa mite in some bee strains.

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BEHAVIORAL RESPONSES OF THE PARSNIP WEBWORM TO HOST PLANT VOLATILES

MARK J. CARROLL* and MAY R. BERENBAUM

Department of Entomology, 320 Morrill Hall
University of Illinois
505 S. Goodwin Ave.
Urbana, Illinois 61801, USA

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Abstract—Sixth instars of the parsnip webworm, *Depressaria pastinacella*, orient by olfaction to bud, male flowers, and female flowers of their primary host plant, *Pastinaca sativa*. Because octyl acetate and octyl butyrate are characteristic of tissues consumed by the sixth instar, we investigated the influence of these esters on webworm feeding behavior and chemo-orientation. Although octyl acetate and octyl butyrate are feeding deterrents, and octyl butyrate is an olfactory repellent, octyl acetate serves as an olfactory attractant. In olfactometers, webworms do not show a preference when given a choice between octyl acetate and host plant tissues. These findings suggest that octyl acetate is a sufficient cue for olfactory orientation. Such behavior may explain differences in the relative abundance of these esters observed among populations of wild parsnip under differential selection pressure from these insects.

Key Words—Olfaction, gustation, attractant, deterrent, volatiles, *Depressaria pastinacella*, *Pastinaca sativa*, Lepidoptera.

INTRODUCTION

The process of host plant location and evaluation is particularly important to insects that consume specific tissues in a narrow range of host species. The parsnip webworm, *Depressaria pastinacella* (Lepidoptera: Elachistidae), is an introduced oligophagous moth effectively restricted throughout much of its range in eastern North America to its introduced host plant wild parsnip, *Pastinaca sativa* (Berenbaum et al., 1986). Later instars feed exclusively upon the buds, flowers, and immature fruits of wild parsnip. Suitable tissues situated within an umbel are

* To whom correspondence should be addressed. E-mail: mcarroll@uiuc.edu

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enclosed by silk; the resulting web contains most of the food required by the larvae, which feed within the web until prepupal wandering commences.

Despite an apparently sedentary larval lifestyle, webworm larvae must be able to locate spatially and temporally ephemeral host tissue. Finding the appropriate site for web construction on a host plant is the task of the larva and not the female moth, in that oviposition occurs in spring several weeks before the appearance of reproductive tissues (Gorder and Mertens, 1984). As a result, a female moth cannot directly assess the suitability of the reproductive tissue that her larvae will eventually consume. Eggs are laid on the underside of leaves, and neonate larvae move individually to axillary meristem tissue (Berenbaum and Zangerl, 1992). Even an established web site may not guarantee an adequate food source. Later instars may become physically displaced from or abandon a web site. Individuals may be ousted by conspecifics seeking to usurp an energetically valuable web site (Green et al., 1998). On occasion, later instars may exhaust the food supply within the confines of the web and be forced to locate new plant material. Later instars show both host-searching behavior and mobility between plants (Berenbaum and Zangerl, 1992). The ability to choose a new site may be important for a webworm, because sites vary in their nutritional suitability (Berenbaum et al., 1984, 1989). The ability to obtain information about host tissue location and condition may therefore, be useful to the caterpillar.

As potential volatile cues, wild parsnip contains a variety of essential oil components, including terpenoids, aliphatic fatty acid derivatives, and phenylpropanoids (Berenbaum and Neal, 1985; Zangerl et al., 1997). Of these, the aliphatic esters octyl acetate and octyl butyrate could provide webworms with highly specific information about host plant tissues. Appreciable biosynthesis of octyl acetate and octyl butyrate is taxonomically restricted to a few genera of apiaceous plants (Bicchi et al., 1990), two of which, *Pastinaca* and *Heracleum*, are the principal host plants of the parsnip webworm (Berenbaum and Zangerl, 1991). In these plants, these two esters are the only essential oil components found exclusively within tissues consumed by the webworm; whereas other essential oil components are distributed throughout the plant, octyl acetate and octyl butyrate are produced only in the reproductive tissues (Zangerl et al., 1997).

Given the site-specificity of octyl acetate and octyl butyrate production, we investigated whether these esters serve as kairomones for sixth instars at concentrations found in the plant. As reliable indicators of suitable host tissue, i.e., reproductive structures, these esters could potentially serve as attractants. Conversely, they may be avoided by webworms due to their co-occurrence and correlation with toxic, but nonvolatile, furanocoumarins in the vittae (oil glands) (Carroll et al., 2000). We conducted a series of studies to determine the behavioral effects of these compounds on webworm larvae. Because gustatory responses can be highly specific with regard to compound and concentration, we tested each compound separately. If webworms do show separate olfactory or gustatory responses to

different esters and behave accordingly, production of esters in parsnip plants, which is genetically variable, could be subject to differential selection pressure from these insects (Berenbaum et al., 1986).

METHODS AND MATERIALS

Insects and Plants. The parsnip webworms used in feeding preference and olfactory tests were collected from two different populations in order to obtain a continuous supply of larvae of an appropriate developmental stage. The parsnip webworms used in the feeding preference tests were collected as fourth and fifth instars on wild parsnip growing in the University of Illinois Phillips Tract, located 6 km northeast of the UIUC campus (Champaign County, Illinois, USA). The parsnip webworms used in the olfactory tests were collected as fourth and fifth instars on wild parsnip growing along Wisconsin State Rd. 60, located 1.2 km east of Lodi (Columbia County, Wisconsin, USA). Although phenologically displaced, these two sites are similar. Both old-field sites included several thousand wild parsnip plants that experienced high levels of herbivory by the parsnip webworm. The larvae were transferred to a semidefined artificial diet (Nitao and Berenbaum, 1988) and raised at approximately 18°C under a 16L:8D photoperiod produced by Phillips 40W Cool White fluorescent bulbs. Parsnip plants used in the olfactory tests were collected from Phillips Tract and transferred in pots to a greenhouse.

Feeding Preferences. Sixth instar feeding preferences for different compounds at a range of concentrations were tested under similar protocols, save for diet treatment and the length of observation period. Octyl acetate and octyl butyrate were obtained from ICN Pharmaceuticals (New York, New York, USA); in an analysis of each ester by GC (Hewlett-Packard 5890), no additional compound peaks were detected. In the first experiment, sixth instars were given a pairwise choice between two diet portions consisting of either (1) a diet supplemented with octyl acetate (0.16%), (2) a diet supplemented with octyl butyrate (0.43%), or (3) an unsupplemented control diet. In the second experiment, the pairwise choice was between two diet portions consisting of either (1) a diet supplemented with average concentrations of octyl acetate (0.16%) and octyl butyrate (0.43%), (2) a diet supplemented with low concentrations of octyl acetate (0.016%) and octyl butyrate (0.043%), or (3) an unsupplemented control diet. These diet concentrations represent the range of phenotypic variation present in wild parsnip fruit (Carroll et al., 2000). For all tests, two diet cubes weighing approximately 1.5 g each were placed on opposite sides of a 12-cm glass Petri dish arena. One recently molted sixth instar was introduced into each Petri dish and allowed to feed *ad libitum*. Because webworms enclose their food with silk and remain to feed, we used caterpillar location as an indicator of feeding preference. The location with respect to the two cubes was scored at 12, 24, and 48 hr in the first experiment and at 1, 2, 6, 12, and 48 hr in the second experiment. If a caterpillar was not on a diet cube, the preference was scored as neutral. The preference ratios obtained at each

observation time were compared by a paired sign test. Neutral preferences were not included in the analysis.

Olfactory Preferences. In the olfactory bioassays, octyl acetate and octyl butyrate were introduced to larvae in a manner consistent with the damaging of tissues, i.e., as a single release of esters in a large volume. Olfactory choice tests were performed by using an enclosed Y-tube apparatus. A vertical larval holding chamber was formed by firmly securing a Pyrex glass Y tube against a 3-cc plastic Luer-Lock syringe. An artificial climbing surface, consisting of a 2-mm-wide strip of Whatman No. 1 filter paper configured into a Y shape, was placed inside the chamber to give the larva ample traction. The terminal end of each Y-tube arm was fitted with a 1-ml disposable Pipetman tip with the apical end distally oriented. The narrow diameter of the apical tip allowed for the attachment of polyethylene tubing carrying volatiles from a remote source.

Purified chemicals and whole plant tissues in bud, male flower, female flower, and half-filled fruit stages were evaluated as olfactory sources. We did not attempt to use the actual amount of esters present in a single fruit because of the difficulty of handling such small volumes and because larvae do not encounter single fruits in isolation; instead, we used 1000 times the amount found in a single parsnip fruit (Carroll et al., 2000). The esters were absorbed into a 3.0-cm-diam. disk of Whatman No. 1 filter paper. To obtain fresh plant material, one umbel (approximately 1.5–2.0 g) in an appropriate developmental stage was cut from a plant immediately before use and placed in water to reduce dehydration. Each source was placed into a 500-ml Erlenmeyer flask. Airflow from the source to the chamber was contained within a completely enclosed system connected by polyethylene tubing 1/4 inch i.d. Air filtered through activated carbon passed over the volatile source and was then shunted to an arm of the Y tube. Negative airflow was maintained through the system by the attachment of the syringe to a model 500× Shop-Vac (Shop Vac Corporation, Williamsport, Pennsylvania, USA). Airflow was adjusted to 0.3 m/sec in the larval chamber with the use of a rheostat. Five chambers were run in parallel, all sharing common volatile sources and negative airflow through the use of adjustable gang valves.

In the bioassay, a sixth instar was introduced into the syringe of the chamber with forceps and allowed to move about freely. A preference was scored when the larva touched the pipet tip within an arm. Pilot studies demonstrated that larvae required an average of 10 min to reach the end of an arm after introduction, so larvae were given 20 min to move within the chamber. The preference was scored as neutral if the larva failed to touch either pipet tip within 20 min. Twenty replicates were performed for each pairwise test. Both the glassware and plastic syringes were rinsed between trials twice with acetone, then rinsed twice with Alconox and water, and dried for at least 1 hr in a 60°C oven to remove any remaining volatiles. The preference ratios obtained between each pair of sources were compared by a paired sign test. Neutral preferences were not included in the analysis.

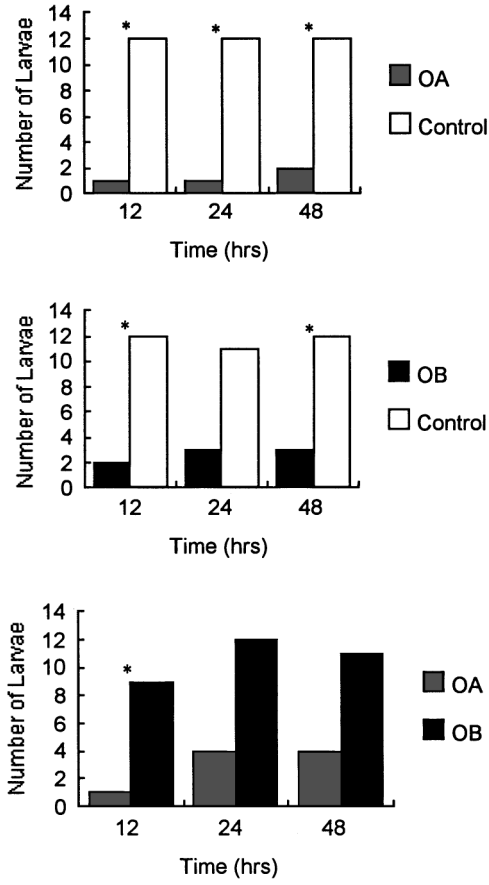


FIG. 1. Feeding preferences of sixth instar *Depressaria pastinacella* toward diets containing either octyl acetate (0.16% OA), octyl butyrate (0.43% OB), or an unsupplemented control diet.

RESULTS

Feeding Preferences. When given a choice, parsnip webworms preferentially fed on octyl ester-free diets rather than on diets containing average levels of either ester (Figure 1). Webworms preferred the control diet over a diet containing octyl acetate during all three observation times (range 12:1 to 12:2; $P < 0.05$, paired sign test). Webworms also preferred the control diet over diets containing octyl butyrate at 12 and 48 hr (range 12:2 to 12:3; $P < 0.05$, paired sign test). In choice

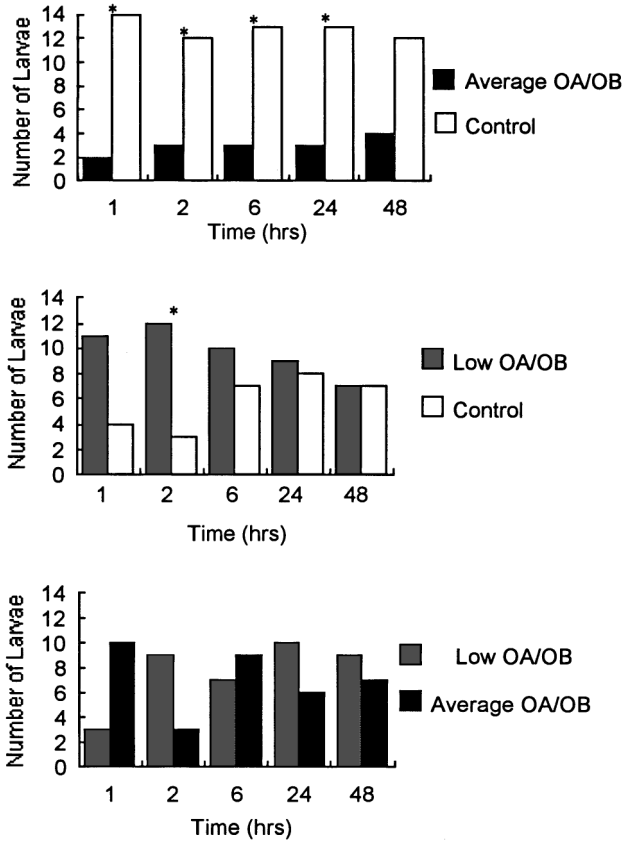


FIG. 2. Feeding preferences of sixth instar *Depressaria pastinacella* toward diets containing different amounts of octyl acetate (OA) and octyl butyrate (OB) (average OA/OB = 0.16% OA and 0.43% OB; low OA/OB = 0.016% OA and 0.043% OB).

tests between the esters, diet containing octyl butyrate was preferred over diet containing octyl acetate at 12 hr (9:1, $P < 0.05$, paired sign test). The same trend was evident at the other observation times, but the differences were not significant, likely because of the sample size used here.

Webworms were also given a choice between diets containing different levels of both octyl esters (Figure 2). The caterpillars preferred the unsupplemented diet over diet containing average levels of the esters at all observation times during the first 24 hr ($P < 0.05$, paired sign test). In contrast, webworms did not consistently prefer unsupplemented diets over diets containing low levels of the esters. The low

TABLE 1. OLFACTORY PREFERENCES OF SIXTH INSTAR *Depressaria pastinacella* TOWARD OCTYL ESTERS PRESENTED IN A Y-TUBE CHOICE ARENA[a]

Comparison	Ratio	P^b
OA vs. control	20:0	<0.001
OB vs. control	1:18	<0.001
OA/OB vs. control	1:19	<0.001
OA vs. OB	20:0	<0.001
OA vs. OA/OB	13:7	0.263
OB vs. OA/OB	4:16	0.012

[a] OA = octyl acetate; OB = octyl butyrate; OA/OB = octyl acetate and octyl butyrate.
[b] Ratio departure from random distribution by a paired sign test (neutral preferences excluded).

ester diet was preferred over the unsupplemented diet only at the 2-hr observation time. Webworms did not distinguish between diets containing average and low levels of octyl acetate and octyl butyrate ($P > 0.05$; paired sign test).

Olfactory Preferences. In the first set of olfactory tests, each octyl ester was compared with a blank control and then with the other ester (Table 1). The webworms preferred octyl acetate over the control but preferred the control over octyl butyrate ($P < 0.001$, paired sign test). When presented with a combination of octyl acetate and butyrate, larval chose the esters over the control ($P < 0.001$; paired sign test). In pairwise comparisons of compounds, octyl acetate was preferred over octyl butyrate ($P < 0.001$; paired sign test). Webworms preferred the ester combination over octyl butyrate ($P = 0.012$) but failed to show a preference between the ester combination and octyl acetate ($P > 0.05$; paired sign test).

In the second set of olfactory tests, parsnip reproductive tissues in different stages of development were evaluated for their olfactory attractiveness (Table 2). In comparisons of plant tissue against a blank control, webworms preferred buds, male flowers, or female flowers over the control ($P < 0.001$; paired sign test). In contrast, webworms preferred the control over half-filled fruits ($P < 0.001$; paired sign test). Each plant tissue was also compared with the other plant tissues. Webworms preferred buds over half-filled fruits ($P < 0.001$; paired sign test). In all other pairwise comparisons, the webworms showed no preference for one tissue over another ($P > 0.05$; paired sign test).

In the final olfactory tests, the four different plant tissues were compared individually against octyl acetate, octyl butyrate, or a combination of both esters. In all pairwise comparisons, no preference was observed between the tissues and the plant compounds ($P > 0.05$; paired-sign test; data not shown).

TABLE 2. OLFACTORY PREFERENCES OF SIXTH INSTAR *Depressaria pastinacella* TOWARD PARSNIP TISSUES PRESENTED IN A Y-TUBE CHOICE ARENA

Comparison	Ratio	P^a
Buds vs. control	20:0	<0.001
Male flowers vs. control	18:0	<0.001
Female flowers vs. control	20:0	<0.001
Half-filled fruits vs. control	2:18	<0.001
Buds vs. male flowers	13:7	0.263
Buds vs. female flowers	8:12	0.503
Buds vs. half-filled fruits	18:2	<0.001
Male flowers vs. female flowers	11:9	0.824
Male flowers vs. half-filled fruits	14:6	0.115
Female flowers vs. half-filled fruits	12:8	0.503

[a] Ratio departure from random distribution by a paired sign test (neutral preferences excluded).

DISCUSSION

With respect to gustation, sixth instars clearly preferred to consume ester-free diets rather than diets augmented with average levels of octyl acetate or octyl butyrate, although they will consume ester-supplemented diets in the absence of alternative food sources. These octyl esters, therefore, may serve as feeding deterrents to sixth instars at the average concentration found in mature fruits. However, the deterrence value of the octyl esters is lost when the dietary concentrations are reduced to one tenth the average concentration in fruits. The threshold for feeding deterrence is within the phenotypic range of fruit contents observed in a wild population (Carroll et al., 2000). Therefore, the fruits of some parsnip plants may lack the ester content required for deterrence and may sustain greater damage. The octyl ester content of other parsnip reproductive tissues attacked by the webworm varies around the threshold value. The average octyl acetate and octyl butyrate content of axillary meristem tissue and buds lies well below the threshold, but both the male and female flowers can have ester contents above the levels required for the observed deterrence (Zangerl et al., 1997). Differences in octyl butyrate content between tissues may also explain sixth instar preferences for parthenocarpic fruits over filled fruits, despite the fact that the former are less nutritious and result in lower growth rates. The preferred parthenocarpic fruits, among other differences, have significantly lower amounts of octyl butyrate than the filled fruits, which may account for the preferences observed (Cianfrogna et al., 2002). Thus, dietary octyl acetate and octyl butyrate may not fully deter webworm herbivory but may influence the selection of host plant tissues when different types are available.

Sixth instar responses toward the esters differ with respect to olfaction alone. Octyl acetate is an olfactory attractant, whereas octyl butyrate is an olfactory repellent. Because the two compounds co-occur in essential oils, the preference of webworms toward a combination of both esters was tested. When both compounds are present, the attractiveness of octyl acetate overrides the repellency of octyl butyrate. This apparent loss of repellency may occur in part because the response to octyl butyrate is concentration-dependent. In a preliminary experiment, we tested the response of webworm larvae to two different amounts of the esters using a similar olfactory apparatus. We used 1000 times the average content of a single fruit as the low amount and 10,000 times the average as a high amount for each ester. Octyl acetate was an attractant at both concentrations, but octyl butyrate was consistently deterrent only at the high amount.

The excess volumes used here may simulate the high effective aerial concentrations encountered at close range at established feeding sites where many hundreds of flowers or fruits may be present (the terminal umbel, generally the most heavily infested by webworms, may contain 1000 or more fruits) (Berenbaum et al., 1986). Release of these esters from undamaged tissues is greatly minimized by the localization of the esters within the vittae. Feeding by webworm larve disrupts the vittae and spreads the essential oil components, thereby greatly enhancing the volatilization of oil components. Behavioral responses to such a release of esters could be biologically relevant in that octyl acetate and octyl butyrate may provide valuable cues to webworms about feeding damage on host plants. The loss of these esters from damaged tissues could provide an orientation cue to displaced or foraging webworms about the suitability of host tissues, because the effective aerial concentration of esters around damaged tissues decreases over time as these compounds escape into the air.

Sixth instars show an olfactory preference for plant tissues, except for half-filled fruits, over a blank control. The bias against half-filled fruits may be due to their eventual development into and resemblance to mature fruits, reproductive tissues that are not regularly consumed by sixth instars. In our field plots, half-filled fruits develop into unpalatable mature fruits within a period of three–seven days. An early sixth instar that selected and webbed together an umbel containing half-filled fruits may therefore not have enough suitable tissue to complete its development. In addition, webworms may not readily distinguish between half-filled and mature fruits, due to similarities in their contents of volatile octyl esters, terpenes, and phenylpropanoids. The volatile profile of half-filled fruits resembles that of mature fruits more so than that of the buds and flowers of earlier developmental stages (Zangerl et al., 1997). The observed preference for buds over half-filled fruits may be due to the marked contrast in the volatile profiles of these tissues, the earliest and latest stages attacked by webworms.

The similarity in response of sixth instars to individual compounds in the presence of whole plant tissues suggests that the parsnip webworm does not require

a complex mixture of volatiles to orient successfully to suitable host plant tissue. This response pattern shows that host-specific volatile compounds alone are sufficient cues for larval orientation to host plant tissues in a laboratory environment, even in the absence of widely distributed compounds such as green leaf volatiles that are used for olfactory orientation by some other insects (Visser, 1986). The distinctive olfactory responses of webworms to octyl acetate and octyl butyrate suggest a specific olfaction mechanism, because the two compounds differ by only two carbons in the ester moiety. Such a distinction could be achieved either through the use of two different olfactory receptors or differential cross-patterning; however, the loss of octyl butyrate repellency in the presence of octyl acetate or plant volatiles shows that interference occurs in the response to both volatiles.

The olfactory preferences demonstrated here suggest that parsnip webworms could exert selective pressures for increasing octyl butyrate and decreasing octyl acetate in tissues; in at least one population, octyl acetate variation is heritable (Carroll et al., 2000). Substantial variation in octyl ester content has been reported for some wild parsnip populations. In Stahl and Kubeczka's (1979) survey of wild parsnip in Europe, two chemotypes showed a complete loss of octyl acetate production accompanied by a concomitant rise in octyl butyrate production. Both parsnip plants and webworms are native to Europe and have had a long evolutionary association there. In contrast, records of the interaction between these two species in the United States date back less than 150 years (Riley, 1888). If European webworms show chemosensory preferences similar to those of the American populations described here, the acetate-deficient chemotypes in Europe may be less attractive (less apparent *sensu* Feeny, 1976) to one of their primary herbivores, as a result of selective pressure exerted by this insect (Berenbaum et al., 1986).

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BEHAVIORAL AND ELECTROPHYSIOLOGICAL RESPONSES OF NATURAL ENEMIES TO SYNOMONES FROM TEA SHOOTS AND KAIROMONES FROM TEA APHIDS, *Toxoptera aurantii*

BAOYU HAN* and ZONGMAO CHEN

Key Laboratory of Tea Chemical Engineering
of Ministry of Agriculture
Tea Institute of Chinese Academy of Agricultural Sciences
Hangzhou 310008 China

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Abstract—Olfactometer bioassays and electrophysiological studies showed that the lacewing, *Chrysopa sinica*, the aphid parasitoid, *Aphidius* sp., and the coccinellid, *Coccinella septempunctata*, all responded to volatiles from tea aphids, *Toxoptera aurantii*, to hexane or ether rinses of tea aphid cuticles, and to synomones released by aphid-damaged tea shoots, as well as to the tea shoot–aphid complex. Each natural enemy spent more time searching on a filter paper treated with tea aphid honeydew than on a blank control filter paper. The interaction between synomones from aphid-damaged shoots and kairomones from tea aphids enhanced the responses to the plant–host complex. There was a significant, logistic dose–response relationship between the number of natural enemies responding and the odor stimulus concentration. Volatile components from the plant–host complex, obtained by air entrainment, were identified by their mass spectra and retention times and confirmed by comparison with standard samples. These were (*Z*)-3-hexen-1-ol, benzaldehyde, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, ocimene, linalool, geraniol, indole, and (*E*)-2-hexenoic acid. The main components in a hexane rinse from tea aphid cuticle were benzaldehyde, undecane, 2,5-hexanedione, 2,5-dihydrothiophene, linalool, 4-methyl-octane, and eicosane, whereas the main components from an ether rinse were (*E*)-2-hexenoic acid, heptadecane, pentadecane, eicosane, tetratetracontane, and nonadecane. Benzaldehyde elicited the strongest responses from natural enemies in the olfactometer and the largest electroantennogram (EAG) responses. While the amount of odor was small, *Coccinella septempunctata* was slightly more sensitive than *Chrysopa sinica* and *Aphidius* sp. An increase in doses of benzaldehyde, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate caused the EAG responses of each natural enemy to decrease. When the doses of (*Z*)-3-hexen-1-ol, linalool, and geranoil

* To whom correspondence should be addressed. E-mail: han-insect@263.net

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increased, EAGs of *Chrysopa sinica* and *Aphidius* sp. increased, but EAGs of *Coccinella septempunctata* decreased. When the dose of indole increased, EAGs of *Coccinella septempunctata* decreased, but those of *Aphidius* sp. increased. This study demonstrates that tea shoot–aphid complexes emit volatile synomones, while the odors from tea aphids, aphid cuticle extracts, and tea aphid honeydew contain kairomones, to which the natural enemies show a logistic dose–response.

Key Words—*Toxoptera aurantii*, *Chrysopa sinica*, *Aphidius* sp., *Coccinella septempunctata*, tea shoot, kairomones, synomones, honeydew, cuticle rinse, behavioral responses, electrophysiology.

INTRODUCTION

Tea is a major crop in China and the high grade teas are processed using the young shoots of the plant. However, the tea aphid, *Toxoptera aurantii* (Boyer) (Homoptera: Aphididae), occurs throughout the Chinese tea region and causes direct feeding damage to tea shoots. In addition, the excreted honeydew on the leaves may be colonized by mildew, which reduces photosynthesis. In Chinese tea gardens, generally, the young tea shoots sprout four or five times a year and are always colonized by populations of tea aphid (Han, 2002). Pesticides are usually not applied to suppress this pest, to avoid residues in the commercial tea, especially in Chinese spring tea. Therefore, the pest can seriously reduce tea output and lower tea quality. The pest also damages tea plants in Japan, India, and Kenya (Das and Kakoty, 1992; Sudoi et al., 1996; Sudoi and Rotich, 1997). In China, larvae and adults of the ladybeetle, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), are voracious predators of tea aphids; in the laboratory an adult can consume 100–120 aphids per day. The population of *C. septempunctata* closely follows the population of the tea aphid, especially from late April to June and from August to October. Larvae and adults of the lacewing,*Chrysopa sinica* Tjeder (Neuroptera: Chrysopidae), are also important predators of tea aphids. The lacewings largely occur in May and from September to October, and an adult can consume 70–90 tea aphids per day under laboratory conditions. The aphid parasite, *Aphidius* sp. (Hymenoptera: Braconidae: Aphidiinae), also attacks both nymphal and adult aphids. The average percentage of parasitism is around 10% in May–June and about 15% in September–October. The pattern of parasitization sometimes appears to be highly aggregated, with parasitism on groups of adjacent tea branches reaching over 90% (Han, 2002).

Several studies have demonstrated that specific blends of odors produced by herbivore-injured plants as well as those released by the pests themselves are often attractive to certain insect predators and parasitoids (Vet and Dicke, 1992; Dicke, 1994). If the semiochemicals were isolated, identified, and synthesized, we could use them to mediate natural enemies controlling tea aphids. Some attempts have been made to use synomones and kairomones in the manipulation of natural

enemies to suppress pests (Bakthavatsalam and Singh, 1996). However, little is known about the chemical communications among insects in tea gardens, and our understanding of the communication mechanisms between pests and predatory insects is poor (Royer and Boivin, 1999). There are limited reports on chemical communication between pest species and their natural enemies. This study examines the origin and the activity of tea aphid-kairomones and synomones from aphid-damaged tea shoots and their use by various natural enemies, and identifies the active components in these infochemicals.

METHODS AND MATERIALS

Preparation and Analysis of Odors

Volatiles from Tea Shoot–Aphid Complexes. Each of the tea shoot–tea aphid complexes was about 7 cm long with one bud and three leaves, weighing appropriately 0.7 g; it supported about 80–150 tea aphids. Two hundred grams of tea shoot–aphid complexes were placed in a 20 l glass vessel with an inlet and an outlet that was modified according to Turlings et al. (1998). The complexes were replaced at 12-hr intervals. Dried and purified air was guided through the inlet at 250–300 ml/min for 48 hr. A Tenax TA column with 150 mg absorbent (Supelco Co.) activated at 275°C for 4 hr with a nitrogen stream was attached to the outlet. A pump was joined to the column and drew at a flow of 250–300 ml/min for 48 hr. Volatiles were desorbed by eluting the tenax traps with 1 ml freshly distilled hexane. Then 20 μl of a 10^{-4} g/ml decanoic acid ethyl ester were added as an internal standard. The resulting extract was concentrated to about 20 μl under a stream of nitrogen, 2 μl of which were immediately injected into a GC-MS. The gas chromatograph was an HP-5890A model, fitted with a PEG-20M quartz capillary column (30 m, 0.2 mm ID), and the mass spectrometer was the HP-5972 MSD model. The GC oven was maintained at 50°C for 5 min and then programmed to increase at 2°C/min to 250°C. Identifications were confirmed by retention time and peak enhancement on co-injection with authentic commercial samples.

A second 1-ml eluting solution of the volatiles from the same weight of tea shoot-aphid complex, collected by air entrainment, was prepared for behavioral bioassays.

Hexane and Ether Rinses of Tea Aphid Cuticle. About 5000 tea aphids of mixed ages were extracted with 5 ml distilled hexane for 60 sec at room temperature as described by Liepert and Dettner (1996). Ten microliters of a 10^{-4} g/ml decanoic acid ethyl ester were added as the internal standard, then the hexane rinse was concentrated to 100 μl under a gentle stream of nitrogen. Substances were tentatively identified by GC-MS as described above. An ether rinse of the same number of tea aphids was also prepared and analyzed.

A further 1-ml hexane rinse and 1-ml ether rinse of the same number of tea aphids were prepared for behavior bioassays, respectively.

Y-Tube Test for Behavioral Responses of Three Species of Natural Enemies to Various Sources of Infochemicals

Insects and Y-Tube Olfactometer Test Procedure. Tea aphids, *Toxoptera aurantii*, and their natural enemies were all collected from tea gardens. *Chrysopa sinica* and *Coccinella septempunctata* were reared on tea aphids in 500-ml glass containers and were starved for 24 hr before use in behavior tests. The parasitoid, *Aphidius* sp., was reared on colonies of tea aphids. In preparation for the tests, female parasitoids that had emerged from mummies isolated in glass vials were mated within 24 hr of emergence and were not allowed to lay eggs or to have any experience with host-related odors. They were also starved for 24 hr before being used in behavior tests. Each individual natural enemy was tested only once in the bioassays.

The Y-tube olfactometer was modified from that described by Du et al. (1996). The base and arms of the olfactometer were each 10 cm long and the angle between the two arms was 120°. The internal diameter was 1 cm, and each arm was connected to either a glass jar containing an odor source or an empty control jar. An electric pump connected to the base of the Y-tube drew air through a flowmeter, an activated charcoal filter, and a molecular sieve before entering the Y-tube so that it was purified of any contaminating odors. The airflow through each of the arms was maintained at 100 ml/min. A smoke test showed that the air of the odor arm did not mix with that of the control arm at the Y junction. Twenty individuals of each natural enemy were tested, one at a time, against each odor source. Each individual was presented with a choice between the test odor and a blank control. The olfactometer arm chosen by the insect after a 5-min period was recorded. After every 10 individuals tested, the Y-tube was cleaned inside and out with pure ethanol, and the treatment arms were reversed to avoid position effects. A χ^2 test was used to determine significant differences between numbers of natural enemies choosing different odor treatments.

Dose Responses. In each test, the control was clean air. The following odor sources were tested, each at eight or nine different concentrations. Each tea shoot used was approximately 7 cm long and had one bud and three leaves. Aphid-damaged shoots were tested immediately after being collected from the tea garden. The regression relationships between the number of natural enemies responding to an odor source (Y) and the concentration of the odor source (X) were calculated using the statistical software package DPS (Tang and Feng, 2000).

The tea shoot-aphid complexes tested included four aphid-damaged shoots with 200 aphids, 10 shoots with 400 aphids, 15 shoots with 600 aphids, 20 shoots with 800 aphids, 30 shoots with 1200 aphids, 50 shoots with 2000 aphids,

70 shoots with 2800 aphids, and 90 shoots with 3600 aphids. The aphid-damaged tea shoots tested included 5, 10, 20, 40, 60, 80, 100, and 120 shoots from which all aphids had been removed. The tea aphids tested included 200, 250, 500, 1000, 1500, 2000, 2500, 3000, and 3500 complexes of adults and nymphs.

Choice Tests. The following combinations of odor sources were presented in olfactometer choice tests to determine which odor was more attractive: (1) 20 aphid-damaged tea shoots with about 1000 aphids vs. 20 undamaged tea shoots, (2) 20 aphid-damaged tea shoots from which the aphids had been removed vs. 20 undamaged tea shoots, (3) 20 aphid-damaged tea shoots with about 1000 aphids vs. 20 aphid-damaged tea shoots from which the aphids had been removed, (4) hexane rinse of aphid cuticle vs. hexane, (5) ether rinse of aphid cuticle vs. ether, (6) the main components identified from volatile extracts of tea shoot-aphid complexes vs. hexane control: (*Z*)-3-hexen-1-ol, benzaldehyde, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, ocimene, linalool, geraniol, indole, and (*E*)-2-hexenoic acid were, respectively, diluted in hexane and used as odor sources at a concentration of 10^{-4} g/ml. In each case, 1 ml of the odor solution was poured into a 2-ml ampoule, which was then placed, unsealed, in the odor source jar. At the same time, 1 ml hexane was poured into an open ampoule and placed in the control jar.

Searching Responses of Natural Enemies to Aphid Honeydew. A Petri dish was used to collect the honeydew secreted by 8000 tea aphids for 24 hr. Five milliliters of a 50% ethanol solution was poured into the dish and stirred for 10 min. The resulting solution was then concentrated to 2 ml. A 12-cm-diam. filter paper was placed into a clean Petri dish, and 100 μl or 200 μl of concentrated honeydew solution were pipetted onto the center of the paper. This was equivalent to the amounts excreted by 400 or 800 aphids over 24 hr, respectively. After the ethanol had evaporated, a natural enemy was introduced in the center of the paper. The time that *C. sinica* and *Aphidius* sp. spent from touching the center to flying off and the time that *C. septempunctata* spent from touching the center to climbing the edge of the dish were recorded. Fifteen individuals of each species were tested. The bioassays were conducted between 09:00 and 14:00 hr at 21–24°C, 75–83% relative humidity and 3200–3600 lux. The tea plant variety was Longjing 43.

Electrophysiology. Electroantennogram (EAG) recordings from each species of natural enemy were made using Ag–AgCl glass electrodes filled with Ringer's solution. The antenna was excised and penetrated with the reference electrode. The tip of the antenna was cut and placed inside the recording electrode. Odor presentation was similar to that in previous studies (Visser, 1979; Park et al., 2000; Han et al. 2001). Twenty microliters of standard solutions of each test compound were applied to filter paper strips (5 × 60 mm), and solvent was allowed to evaporate for 30 sec before the paper strip was inserted into the glass Pasteur pipet (15 cm long). The tip of the pipet was inserted about 3 mm into a small hole in the wall of a steel tube (15 mm diam. × 15 cm long) directed over the antennal

preparation. An air stimulus controller (model CS-05b, Syntech) was used for air and odor delivery with a constant flow (120 ml/min) of charcoal-filtered and humidified air passed over the antenna through the open end of the steel tube positioned 15 mm from the antenna. During odor stimulation, 20 ml/min of air were applied through the pipet into the main airflow for 0.5 sec. Intervals of at least 2 min were maintained between stimulations, which were delivered in ascending order of concentration. Compounds were tested against 15 individual antennae. The signals were passed through an amplifier (Syntech CS-05), displayed on an oscilloscope and stored in a computer using Syntech Software.

The odor sources were: (1) air entrainment extracts of tea shoot–aphid complexes (TSAC), air entrainment extracts of undamaged tea shoots (UTS), hexane rinse, and ether rinse; (2) benzaldehyde, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexen-1-ol, linalool, geranoil, and indole diluted in hexane at concentrations of 10^{-2}, 10^{-4}, and 10^{-6} g/ml; and (3) filter paper that contained honeydew from 800 tea aphids excreted for 24 hr (the filter paper was cut into strips 60 mm long $\times$ 5 mm wide to fit in the odor delivery glass Pasteur pipet).

RESULTS

Chemical Composition of Several Volatile Infochemicals. Nine components were confirmed in the volatile extract entrained from tea shoot–aphid complexes. (*Z*)-3-Hexen-1-ol, geraniol, (*E*)-2-hexenal, and benzaldehyde were present in the greatest quantities (Table 1). The hexane and ether rinses of aphid cuticle contained many hydrocarbons among their main components. Benzaldehyde was a major component in the hexane rinse (Table 2).

TABLE 1. VOLATILE COMPOSITION OF TEA SHOOT–APHID COMPLEXES AND RELATIVE AMOUNTS

Component	Amount[a]
(*Z*)-3-hexen-1-ol	8.80×10^{-6}
geraniol	5.40×10^{-6}
(*E*)-2-hexenal	4.20×10^{-6}
benzaldehyde	3.30×10^{-6}
ocimene	2.24×10^{-6}
Decanoic acid, ethyl ester (internal standard)	2.00×10^{-6}
linalool	1.20×10^{-6}
(*Z*)-3-hexenyl acetate,	0.24×10^{-6}
(*E*)-2-hexenoic acid	0.20×10^{-6}
indole	0.04×10^{-6}

[a] Units: g/20 μl concentrated resulting extract.

TABLE 2. MAIN COMPONENTS OF HEXANE AND ETHER RINSES OF APHID CUTICLE AND THEIR RELATIVE AMOUNTS[a]

Component	Hexane rinse	Ether rinse
2,5-Hexanedione	1.63×10^{-5}	
Benzaldehyde	6.90×10^{-6}	
linalool	6.50×10^{-6}	
2,5-Dihydro-thiophene	5.00×10^{-6}	
Eicosane	3.50×10^{-6}	2.41×10^{-5}
Undecane	2.95×10^{-6}	
Decanoic acid, ethyl ester (internal standard)	1.00×10^{-6}	1.00×10^{-6}
4-Methyl-octane	0.35×10^{-6}	
2-Hexenoic acid		4.80×10^{-5}
Heptadecane		3.50×10^{-5}
Pentadecane		4.00×10^{-6}
Tetratetracontane		2.90×10^{-6}
Nonadecane		0.50×10^{-6}

[a] Units: g/100 μl concentrated rinse.

Dose Responses of Natural Enemies to Tea Shoot–Aphid Complexes. All three natural enemies responded to tea shoot–aphid complexes in the Y-tube olfactometer, and the number responding and the speed of their response increased as the amount of the odor source increased (Table 3). In the case of *C. sinica*, the difference between the number of natural enemies choosing odor and the number choosing control became statistically significant when the odor source was 50 aphid-damaged shoots with 2000 tea aphids ($P < 0.05$). The level of significance increased when the odor source was at least 70 aphid-damaged shoots

TABLE 3. NUMBER OF NATURAL ENEMIES CHOOSING TEA SHOOT–APHID COMPLEXES OR BLANK CONTROL IN A Y-TUBE OLFACTOMETER[a]

Number of aphids/100	(X_1)	2	4	6	8	12	20	28	36
Number of aphid-damaged shoots	(X_2)	4	10	15	20	30	50	70	90
Number of natural enemies choosing odor (Y) or control									
Chrysopa sinica	Odor (Y)	10	11	12	13	13	15*	17**	19**
	Control	10	9	8	7	7	5	3	1
Aphidius sp.	Odor (Y)	10	10	11	11	12	13	15*	17**
	Control	10	10	9	9	8	7	5	3
Coccinella septempunctata	Odor (Y)	10	12	13	14	15*	17**	18**	19**
	Control	10	8	7	6	5	3	2	1

[a] Notes: χ^2 test is used to determine the difference between number of natural enemies choosing odor and number of choosing control. * and ** indicate significances statistical at $P < 0.05$ and $P < 0.01$, respectively.

with 2800 aphids ($P < 0.01$). There was a highly significant linear regression relationship between the number of natural enemies choosing odor (Y) and the number of aphids/100 (X_1) as well as the number of aphid-damaged shoots (X_2). The relationship can be represented as $Y = 10.4618 - 2.0938\,X_1 + 0.9314\,X_2$ ($R^2 = 0.9899$; $P < 0.001$).

For *Aphidius* sp., the difference between the number of natural enemies choosing odor and the number choosing control became statistically significant only when the odor source was 70 aphid-damaged shoots with 2800 aphids ($P < 0.05$). Again, there was also a highly significant linear regression relationship ($Y = 9.3855 + 0.7186\,X_1 - 0.2057X_2$; $R^2 = 0.9881$; $P < 0.001$).

For *C. septempunctata*, the difference between the number of natural enemies choosing odor and the number of choosing control became statistically significant when the odor source was 30 aphid-damaged shoots with 1200 aphids ($P < 0.05$), and there was a highly significant linear regression relationship ($Y = 11.9787 - 5.7940X_1 + 2.4023X_2$; $R^2 = 0.9693$; $P < 0.001$).

The number of both aphids and aphid-damaged shoots closely corelates with the number of natural enemies locating odor. Therefore, the volatiles emitted from both aphids and aphid-damaged shoots can simultaneously seduce natural enemies. However, the absolute values of coefficients of X_1 and X_2 regarding *C. septempunctata* equation are the largest, which means that both odors attract *C. septempunctata* a little more strongly than the other natural enemies. In fact, when the amount of the odor source is small, *C. septempunctata* is slightly more responsive than *C. sinica* and *Aphidius* sp. (Table 3).

Dose Responses of Natural Enemies to Aphid-Damaged Tea Shoots. Aphid-damaged tea shoots were also attractive to the three natural enemy species (Table 4). For *C. sinica*, the difference between the number natural enemies choosing odor and the number choosing control became statistically significant when the odor source was 60 aphid-damaged shoots ($P < 0.05$). The significance level increased when the odor source was at least 80 aphid-damaged shoots

TABLE 4. NUMBERS OF THREE SPECIES OF NATURAL ENEMIES CHOOSING APHID-DAMAGED TEA SHOOTS OR BLANK CONTROL IN A Y-TUBE OLFACTOMETER[a]

Number of aphid-damaged shoots	(X)	5	10	20	40	60	80	100	120
Number of natural enemies choosing odor (Y) or control									
Chrysopa sinica	Odor (Y)	10	10	12	13	15*	17**	18**	18**
	Control	10	10	8	7	5	3	2	2
Aphidius sp.	Odor (Y)	10	10	12	13	14	15*	16*	18**
	Control	10	10	8	7	6	5	4	2
Coccinella septempunctata	Odor (Y)	10	11	14	16*	16*	17**	18**	18**
	Control	10	9	6	4	4	3	2	2

[a] *and **indicate significance levels of $P < 0.05$ and $P < 0.01$, respectively.

($P < 0.01$). There was a significant logistic regression relationship between the number of natural enemy responding (Y) and the number of aphid-damaged shoots (X), which can be represented as $Y = 19.8737/(1 + e^{0.132-0.022X})$ ($R^2 = -0.9851$; $P < 0.001$). For *Aphidius* sp., the difference became statistically significant when the odor source was 80 aphid-damaged shoots ($P < 0.05$), and there was also a significant logistic regression relationship, $Y = 23.5911/(1 + e^{0.326-0.0117X})$ ($R^2 = -0.9722$; $P < 0.001$). For *C. septempunctata*, the difference became statistically significant when the odor source was 40 aphid-damaged shoots ($P < 0.05$), and again there was a significant logistic regression relationship, $Y = 17.5500/(1 + e^{-0.0287-0.0561X})$ ($R^2 = -0.9678$; $P < 0.0001$).

Logistic responses exist widely in living processes. The responses of natural enemies to odors agree with the model. Similarly, while the amount of the odor source is small, *C. septempunctata* is slightly more responsive than the other two species of natural enemies (Table 3).

Dose Responses of Natural Enemies to Tea Aphids. Tea aphids themselves also send out odors incurring natural enemies (Table 5). In the case of *C. sinica*, the difference between the number of natural enemies choosing odor and the number of choosing control became statistically significant when the odor source was 2000 aphids ($P < 0.05$). The significant logistic regression relationship between the number of natural enemies responding (Y) and the number of aphids/100 (X) could be represented as $Y = 25.6567/(1 + e^{0.5217-0.0405X})$ ($R^2 = -0.9923$; $P < 0.001$). For *Aphidius* sp., the difference became statistically significant when the number of aphids was 2500 ($P < 0.05$), and there was a significant logistic regression relationship that could be represented as $Y = 21.5912/(1 + e^{0.2006-0.0413X})$ ($R^2 = -0.9975$; $P < 0.001$). For *C. septempunctata*, the difference became statistically significant when the number of aphids was 2000 ($P < 0.05$). The significant logistic regression relationship could be represented as $Y = 23.2074/(1 + e^{0.1779-0.0457X})$ ($R^2 = -0.9079$; $P < 0.01$).

TABLE 5. NUMBER OF THREE SPECIES OF NATURAL ENEMIES CHOOSING APHIDS OR BLANK CONTROL IN A Y-TUBE OLFACTOMETER

Number of tea aphids/100	(X)	2	5	10	15	20	25	30
Number of natural enemies choosing odor (Y) or control								
Chrysopa sinica	Odor (Y)	10	11	12	13	15*	16*	17**
	Control	10	9	8	7	5	4	3
Aphidius sp.	Odor (Y)	10	11	12	13	14	15*	16*
	Control	10	9	8	7	6	5	4
Coccinella septempunctata	Odor (Y)	10	13	14	14	15*	17**	18**
	Control	10	7	6	6	5	3	2

[a] * and ** indicate significance levels of $P < 0.05$ and $P < 0.01$, respectively.

Selectivity of Natural Enemies to Different Odor Sources in Choice Tests. The volatiles from both tea shoot–aphid complexes and aphid-damaged tea shoots were much more attractive to natural enemies than the volatiles from undamaged tea shoots. Furthermore, the volatiles from tea shoot–aphid complexes were slightly more attractive than the volatiles from aphid-damaged shoots, possibly because the interaction between aphids and aphid-damaged shoots strengthens the attraction (Table 6). Hexane and ether rinses of tea aphid cuticles showed some attractive effect on natural enemies. The hexane rinse was slightly more attractive than the ether rinse.

Of nine components identified from the volatile extracts entrained from tea shoot–aphid complexes, statistically significant responses of the natural enemies were recorded only to benzaldehyde and (*E*)-2-hexenal ($P < 0.05$). The responses to benzaldehyde were slightly stronger than those to (*E*)-2-hexenal.

Attraction of Aphid Honeydew to Natural Enemies. All three natural enemy species spent significantly longer searching on filter papers with honeydew than on blank control filter papers ($P < 0.01$) (Table 7). On the honeydew-treated filter paper, the lacewing and the coccinellid actively searched with much turning and reduced walking speeds. They still moved their antennae from side to side and shook their labial palps constantly. The parasitoid exhibited arrestment, antennal examination, and ovipositor probing. Therefore, the honeydew intensively entices natural enemies and appears to provide an important contact semiochemical cue for natural enemies to search tea aphids.

Electrophysiology. EAG responses of three species of natural enemies elicited by air entrainment volatile extracts from tea shoot–aphid complexes were significantly greater than those elicited by extracts from undamaged shoots. Hexane and ether rinses of aphid cuticles also elicited strong EAG responses. EAG responses of *C. septempunctata* and *C. sinica* to these infochemicals were much larger than those of *Aphidius* sp. (Figure 1). In particular, honeydew vividly elicited EAG responses from *C. septempunctata*, which suggests honeydew contains semiochemicals.

Of seven main components in the volatile extracts of tea shoot–aphid complexes, benzaldehyde elicited the biggest EAG responses, followed by (*E*)-2-hexenal and (*Z*)-3-hexenyl acetate. EAG responses of the three species of natural enemies decreased when the dose of the three components increased. When the dose of linalool, geraniol, and (*Z*)-3-hexen-1-ol increased, EAGs of *C. sinica* and *Aphidius* sp. increased, but EAGs of *C. septempunctata* gradually decreased. When the dose of indole increased, EAG responses of *C. septempunctata* decreased, but those of *Aphidius* sp. increased (Figure 2). EAG responses of the predatory or parastic natural enemies to infochemicals appear to change as the concentrations of infochemicals increase or decrease. The numerical values of EAG of one natural enemy differ from those of another. EAGs of *C. septempunctata* are the greatest, and those of *Aphidius* sp. are the smallest.

TABLE 6. DIFFERENCE IN NUMBER FOR NATURAL ENEMIES CHOOSING DIFFERENT ODOR SOURCES IN CHOICE TESTS IN Y-TUBE OLFACTOMETERS[a]

	Odor			Odor			Odor			Odor			Odor		
Natural enemy	TSAC	UTS	Difference	ADS	UTS	Difference	TSAC	ADS	Difference	HR	H	Difference	ER	E	Difference
C. sinica	16	4	*	15	5	*	12	8	NS	12	8	NS	11	9	NS
Aphidius sp.	16	4	*	15	5	*	12	8	NS	12	8	NS	11	9	NS
C. septempunctata	16	4	*	16	4	*	12	8	NS	12	8	NS	11	9	NS

[a] Notes: TSAC: tea shoot-aphid complexes; UTS: undamaged tea shoots; ADS: aphid-damaged shoots; HR: hexane rinse; H: hexane; ER: ether rinse; E: ether; NS: No significant. χ^2 test is used to determine the difference between the number for natural enemies choosing one odor and the number for natural enemies choosing another odor resource in choice tests. * indicates statistical significance of $P < 0.05$.

TABLE 7. DIFFERENCE BETWEEN SEARCHING TIMES (SECONDS) OF NATURAL ENEMIES ON HONEYDEW-TREATED AND ON BLANK CONTROL FILTER PAPERS[a]

	Control	100 μl	200 μl	Difference
Chrysopa sinica	2.1 ± 0.9	10.7 ± 3.4	25.4 ± 12.2	$F = 38.6 > F_{0.01}(2, 42)$
Aphidius sp.	27.0 ± 13.1	117.0 ± 60.9	200.1 ± 72.5	$F = 28.2 > F_{0.01}(2, 42)$
Coccinella septempunctata	8.6 ± 3.6	47.1 ± 15.9	71.9 ± 24.8	$F = 52.1 > F_{0.01}(2, 42)$

[a] Values are mean $\pm$ SE; $F_{0.01}(2, 42) = 5.15$; 15 replicates.

DISCUSSION

Herbivore-induced plant odors play a major role in the foraging behavior of predatory and parasitic insects. Turlings et al. (1998) reported that the aphid, *Rhopalosiphum maidis*, induced no measurable emissions of volatiles when feeding on maize plants and suggested that the *R. maidis*–maize interaction is different from some other known interactions between plants and aphids. Some of the behavioral studies conducted on aphid parasitoids have indicated that induced plant volatiles are involved (Guerrieri et al., 1993; Reed et al., 1995; Du et al., 1996, 1998; Powell et al., 1998). Even uninfested parts of aphid-infested plants have been shown to be attractive to parasitoids (Micha and Wyss, 1995). The seven-spotted ladybird, *Coccinella septempunctata* L. (Coleoptera, Coccinellidae), responded positively to volatiles from aphid, *Rhopalosiphum padi* (L.), from infested plants, *Hordeum vulgare*, and from previously aphid-infested plants, but not to volatiles

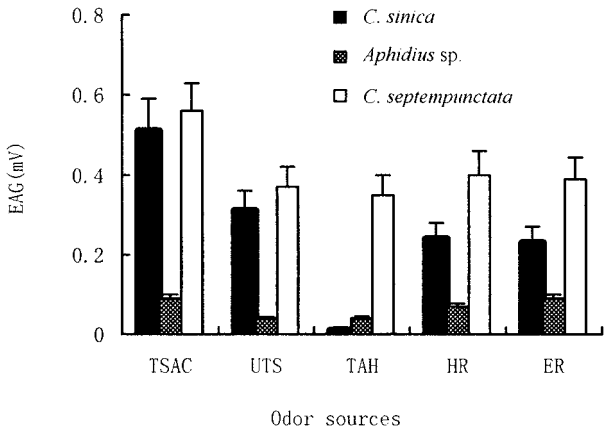


FIG. 1. Electroantennogram responses of three species of natural enemies to the following infochemicals: TSAC: air entrainment volatile extracts from tea shoot-aphid complexes; UTS: air entrainment volatile extracts from undamaged tea shoots; TAH: tea aphid honeydew; HR: hexane rinse of tea aphid cuticles; ER: ether rinse of tea aphid cuticles.

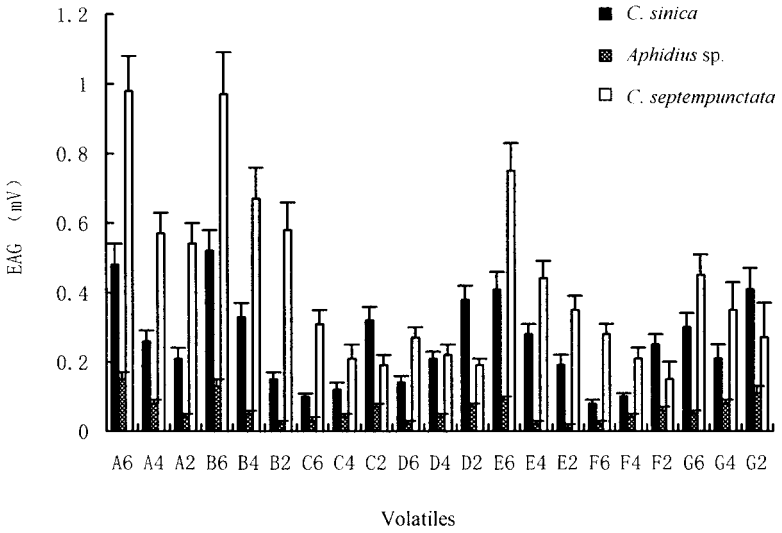


FIG. 2. EAG responses of three species of natural enemies to seven main components in the volatiles entrained from tea shoot–aphid complexes. A6, A4, and A2 stand for 10^{-6}, 10^{-4}, and 10^{-2}g/ml benzaldehyde, respectively. B6, B4, and B2 for 10^{-6}, 10^{-4}, and 10^{-2} g/ml (*E*)-2-hexenal, respectively. C6, C4, and C2 for 10^{-6}, 10^{-4}, and 10^{-2} g/ml linalool, respectively. D6, D4, and D2 for 10^{-6}, 10^{-4}, and 10^{-2} g/ml (*Z*)-3-hexen-1-ol, respectively. E6, E4, and E2 for 10^{-6}, 10^{-4}, and 10^{-2} g/ml (*Z*)-3-hexenyl acetate, respectively. F6, F4, and F2 for 10^{-6}, 10^{-4}, and 10^{-2} g/ml geraniol, respectively. G6, G4, and G2 for 10^{-6}, $10^{-4}$and 10^{-2} g/ml indole, respectively.

from uninfested plants in olfactometer bioassays (Ninkovic et al., 2001). In the present work, we demonstrated that interactions between tea aphids and tea shoots produce volatiles that are attractive to predatory and parasitic natural enemies. The volatiles released by tea shoot-aphid complexes, and the volatiles released by aphid-damaged tea shoots act as long-range cues, while the odors of tea aphids themselves act as short-range cues. The interaction between the kairomones from tea aphids and the synomones from aphid-damaged shoots reinforced the attractive effect of tea shoot–aphid complexes on natural enemies, and allowed them, to some extent, to overcome the "reliability–detectability problem" faced by foraging carnivores (Vet et al., 1991; Vet and Dicke, 1992).

In recent years, aspects of chemical communication via surface lipids and hydrocarbons have been increasingly investigated. Cuticular hydrocarbons play a role as recognition cues in *Apis mellifera carnica* Pollm, allowing recognition of nestmates and conveying information about age, caste, and sex (Fröhlich et al., 2000). The Aphidiine wasp *Lysiphlebus cardui* (Marshall) is the dominant parasitoid of the ant-attended aphid, *Aphis fabae cirsiiacanthoidis* Schrank (Afc), and is

successful because the composition of the cuticular hydrocarbons of the wasp is very similar to that of the ant *Lasius niger* L., allowing *L. cardui* to forage in the aphid colonies without being attacked (Liepert et al., 1996). The hydrocarbons act as chemical mimics. Bakthavatsalam et al. (1999) reported that a hexane wash of the body of *Opisina arenosella* Walker (Lepidoptera: Noctuidae), a major pest of coconut in coastal Karnataka in India, elicited positive responses from its parasitoids, *Goniozus nephantidis* (Muesebeck), *Brachymeria nephantidis* Gahan, and *Elasmus nephantidis* Rohwer in a Y-tube olfactometer. The hexane wash of the larvae of *O. arenosella* had 20 different alkanes, including the five alkanes identified from cuticle rinses of tea aphids, i.e., undecane, eicosane, heptadecane, nonadecane, and pentadecane. The tea aphid cuticle contains many hydrocarbons. Cuticle rinses from tea aphids act as short-range cues or contact semiochemicals for the three natural enemies tested. The hexane rinse of tea aphid cuticle appeared to be slightly more attractive than the ether rinse, and one of the reasons may be that it contained benzaldehyde.

The western flower thrips, *Frankliniella occidentalis* (Pergande), responded to benzaldehyde at a 10% concentration in Y-tube olfactometer bioassays (Koschier et al., 2000). In the field, traps with benzaldehyde were also attractive to western flower thrips (Teulon et al., 1993). Guo et al. (1998) reported that benzaldehyde exists in tea leaves in the form of cyanogenic glycosides, i.e., prunasin, which can be hydrolyzed into benzaldehyde, hydrocyanic acid, and glucose by β-d-glucosidase. The large amounts of benzaldehyde detected in the volatiles entrained from the tea shoot–aphid complexes possibly resulted from enzymes present in the saliva of the aphids, because the oral cavity of many aphids is known to contain β-d-glucosidase (Madhusudhan et al., 1994). Cyanogenic compounds are widely distributed in the plant kingdom (Seigler, 1981) and in several arthropods (Duffey, 1981) and other insects (Davis and Nahrstedt, 1985). These are usually stabilized by glycosylation giving rise to the cyanogenic glycosides or (rarely) by esterification of the hydroxyl function, resulting in the cyanolipids. If a plant or animal tissue is injured, the glycosides are hydrolyzed by glycosidases, and the resulting cyanogenic compounds decompose under the influence of pH or nitrilase activity to a carbonyl component and HCN (Hösei, 1981). This carbonyl component is an aldehyde or ketone, e.g., benzaldehyde in the case of prunasin. The aldehyde often possesses cytotoxic activity, and cyanogenic compounds act as protection agents for plants (Nahrstedt, 1985). Benzaldehyde is the most common benzenoid (Knudsen et al., 1993) and is a minor aromatic component of tea plants. It occurs in tea leaves in trace amounts at levels of about 7.4 mg/kg. However, in the present study, benzaldehyde was detected in higher amounts in the volatiles entrained from aphid-damaged shoots infested with tea aphids. Furthermore, it elicited both strong behavioral responses and electrophysiological responses. We consider it to be an important, aphid-induced component of the volatiles released from tea shoots that influences natural enemy foraging behavior.

The honeydew excreted by the cereal aphid, *Sitobion avenae* (F.), acts as a contact kairomone for the coccinellid *C. septempunctata* (Carter and Dixon, 1984). Honeydew of the rose-grain aphid *Metopolophium dirhodum* (Walker) and of the pea aphid *Acyrthosiphon pisum* (Harris) stimulated the syrphid *Episyrphus balteatus* (Deg.) to lay eggs (Budenberg and Powell, 1992). The wasp *Diaeretiella rapae* (M'Intosh) used honeydew produced by its host, the cabbage aphid *Brevicoryne brassica* (Linnaeus), as a kairomone (Shaltiel and Ayal, 1998). The parasitoid *Aphidius nigripes* Ashmead spent more time searching on potato previously infested with potato aphids, *Macrosiphum euphorbiae* (Thomas) than on fresh plants, while water extracts of the aphid honeydew applied to the surface of foliage or to filter paper disks increased searching time (Bouchard and Cloutier, 1984). Wasps also frequently turned, protracted their abdomen, and tapped the paper with their antennae. Li et al. (1993) identified 12 amino acids and six sugars in honeydew of the citrus white fly, *Aleurocanthus spiniferus* (Quaintance). These constituted an "artificial honeydew," which was sprayed on tea plants in a tea garden, resulting in an increase of 10–20% in parasitism of *A. spiniferus* by *Amithus longiconis* Föster. Seven saccharides and 13 amino acids were identified from the honeydew of tea aphids (Han and Chen, 2000). In the present study, the honeydew had weak volatility and was a searching stimulant to three species of beneficial insects. We also consider that the honeydew acts as an important contact kairomone. EAG responses of *C. septempunctata* to honeydew were stronger than those of *C. sinica* and *Aphidius* sp. This may be because the coccinellids usually crawl up and down on tea plants searching for tea aphids and easily contact honeydew, so they are sensitive to it as a foraging cue.

The present work showed that each natural enemy has its reaction threshold to a semiochemical cue, defined in time and space. When the dose of the odor source increased, the level of response to semiochemicals ascended on a Logistic curve. However, there are differences in electrophysiology and behavioral responses between *C. sinica*, *C. septempunctata*, and *Aphidius* sp. to the same infochemicals. When the dose of the odor source is small, *C. septempunctata* is the most sensitive. When the dose reaches a certain level, all three natural enemies respond equally, for which the mechanism used to receive infochemicals may be responsible.

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RESPONSE TO WALNUT OLFACTORY AND VISUAL CUES BY THE PARASITIC WASP *Diachasmimorpha juglandis*

M. LAWRENCE HENNEMAN,[1,2,*] ERIC G. DYRESON,[1,2] JUNJI TAKABAYASHI,[3] and ROBERT A. RAGUSO[2,4]

[1]*University of Montana-Western*
Department of Environmental Science and Mathematics
710 S. Atlantic Street
Dillon, Montana 59725 USA

[2]*Center for Insect Science*
University of Arizona
Tucson, Arizona 85721, USA

[3]*Center for Ecological Research*
Kyoto University
Otsuka 509-3, Hirano, Kamitanakami, Otsu, 520-2113, Japan;
and CREST, JST (Japan Science and Technology Corporation)

[4]*University of South Carolina*
Department of Biology
Coker Life Sciences Building
Columbia, South Carolina 29208 USA

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Abstract—*Diachasmimorpha juglandis* is a specialist parasitoid attacking fly larvae in the genus *Rhagoletis* that feed exclusively on walnut fruit husks. In a free-foraging assay comparing response to uninfested, infested, and mechanically damaged fruits, we first determined that *D. juglandis* use host feeding damage on the fruit as a cue for host presence. In another free-foraging assay that used artificial walnut models and wind tunnel experiments, *D. juglandis* distinguished infested from uninfested fruits by using either olfactory or visual cues separately. However, the response rate of wasps in the wind tunnel was raised considerably when visual cues were also available. We analyzed the volatile compounds emitted by cohorts of uninfested, mechanically damaged, and infested fruits 1, 3, 5, 8, and 10 days after oviposition by flies into the infested fruits. Total volatile emissions did not differ significantly among treatments, but quantitative changes in volatiles distinguished infested fruits from uninfested and damaged fruits. The fact that parasitoids did not distinguish between

* To whom correspondence should be addressed. E-mail: L_Henneman@umwestern.edu

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infested and damaged fruits in assays where damage was visible indicates that they rely on visual cues when those are available.

Key Words—Hymenoptera, Braconidae, Opiinae, *Biosteres*, *Diachasmimorpha*, parasitoid, foraging, olfactory cues, visual cues, wind tunnel, volatile analysis.

INTRODUCTION

Insect natural enemies are known to increase their host-searching efficiency by using, as indirect cues, odors associated with the plants on which their hosts feed (Read et al., 1970; Price et al., 1980; Whitman and Eller, 1990; Vet et al., 1995). Parasitoids are often attracted to damaged plants, with the highest response to damage created specifically by a feeding herbivore (Eller et al., 1988; Turlings et al., 1990, 1991a; McAuslane et al., 1991). Unlike simple mechanical damage, herbivore damage to foliage can result in systemic release by a plant of specific volatile compounds. (Turlings et al., 1991b; Turlings and Tumlinson, 1992; Dicke et al., 1993; Röse et al., 1996).

Wasps are known to use visual as well as odor cues to locate leaf-feeding hosts (e.g., Sugimoto et al., 1988; McAuslane et al., 1991; Drost and Cardé, 1992; Wäckers, 1994; Wäckers and Lewis, 1994); however, the use of odor has been emphasized in these systems. In systems in which the host is an internally feeding frugivore, studies examining the roles of both odor and visual cues on parasitoid foraging have focused on how the presence of odor mediates response to visual stimuli in parasitoids (Leyva et al., 1991; Messing and Jang, 1992; Messing et al., 1996; Jang et al., 2000). We are interested in specifically what visual and olfactory cues allow parasitoids to distinguish infested from uninfested fruits.

We examine here the foraging behavior of the opiine braconid parasitoid *Diachasmimorpha juglandis*, which attacks fly larvae in the genus *Rhagoletis* that feed inside the husks surrounding walnut fruits. Walnut fruits are yellowish green when ripe. Flies oviposit clutches of 10–20 eggs in fruits when fruits are on the tree and ripe (i.e., soft enough for a fly to insert her ovipositor) (Papaj, 1994). After a fly has oviposited, the fruit begins to appear more yellow. Eggs hatch in ca. 3 days. The natural rotting process of a fruit is accelerated as larvae feed and develop within, and dark brown patches appear over areas in which larvae have consumed the inner husk material (Henneman and Papaj, 1999). *Diachasmimorpha juglandis* relies on indirect cues for locating infested fruits, because the larvae feed internally. In Arizona, nearly all walnut fruits eventually become infested, but early in the season there are both infested and uninfested fruits available.

Diachasmimorpha juglandis females land preferentially on infested walnuts compared to ripe, uninfested walnuts (Henneman, 1996). Here we used behavioral assays and walnut fruit volatile analysis to test the presence of feeding damage as

a cue used by *D. juglandis* for host presence and to evaluate separately the use of olfactory and visual cues by wasps foraging for hosts inside fruits.

METHODS AND MATERIALS

Wasps were collected in the summer of 1994 as larvae within their fly larvae hosts. Infested walnuts were collected from several sites in southeastern Arizona, USA, including Wet Canyon in the Pinaleño Mountains (Graham County), Garden Canyon in the Huachuca Mountains (Ft. Huachuca, Cochise County), the Catalina Mountains (Tucson, Pima County), and Patagonia Square (Patagonia, Santa Cruz County). After host larvae exited the walnuts and pupated in the sand provided, puparia containing both host pupae and wasp larvae were stored at 5–10°C in the dark until the following summer. About 6 wk after warming pupae at ca. 30°C, adult *D. juglandis* emerged. After emergence, male and female *D. juglandis* adults were caged together to allow mating and were maintained with water and sugar at ca. 30°C for at least 5 d to allow egg development in females (Buckingham, 1975). Five- to 14-d-old females were used in all experiments.

Greenhouse Cage Assay—Real Fruits. The purpose of this assay was to determine whether foraging wasps use fruit damage as a cue for hosts. At least 1 d before the experiment began, female wasps were individually marked with 1–2 dots of liquid tempera paint (ProArt) on the thorax. The evening before testing, marked females were placed inside the greenhouse to allow acclimatization to greenhouse conditions.

Most free-foraging fruit choice assays were conducted in a 3-m-diam. × 3-m tall cylindrical field cage inside the greenhouse, which contained three potted walnut trees, each 2–3 m in height. Data were taken between 10:00 and 16:00 hr on various test days from July to November 1995. There were 1 or 2 test periods of 1–3 hr each on a given day. Temperature during tests ranged from 25° to 35°C, and relative humidity ranged from 30% to 60%. Additional data were collected in August 1995 in a field cage enclosing a branch of a walnut tree at the Southwestern Research Station of the American Museum of Natural History, Portal, Arizona, using wasps collected from the field as adults.

Ten to 30 female wasps were released into the cage on a given test day. At most, 7 of these alighted on at least one walnut fruit. Separate groups of wasps were tested in three experiments which paired three different fruit types: host-infested (= infested), uninfested but mechanically damaged (= damaged), and uninfested and undamaged (= uninfested). Mechanically damaged fruits were manually bruised over roughly 20% of the surface of the fruit, using moderately applied thumb pressure, at least 1 hr before an experiment. Within a few minutes after bruising, the skin of the fruit acquired a brown color that closely resembled feeding damage by *Rhagoletis* larvae and similarly got darker over time. This is

a reproducible technique, similar to leaf damage techniques employed by other workers (Turlings et al., 1990; Turlings and Tumlinson, 1992). Fruits were placed 1–2 m high in the cage and at least 0.5 m apart.

In the first experiment, wasps were presented with 4 infested and 4 damaged fruits. In the second, wasps were presented with 4 damaged fruits and 4 uninfested fruits. In the third, they were presented with 4 infested and 4 uninfested fruits. All fruits used at one time were collected from a single tree, and 3–6 sets of fruits from different trees were used in each experiment. Each set of fruits was used over 2–3 d, when damage covered 20–50% of the surface of the infested fruits, after which all fruits were discarded. The position of each fruit was randomized before every test period. Landings made on each fruit were recorded for all wasps by speaking into a hand-held tape recorder.

Greenhouse Cage Assay—Artificial Models. To assess the role of visual cues apart from odor cues in the landing behavior of wasps, an assay similar to that described above was conducted, except that artificial walnut fruit models (rather than real fruits) were presented to wasps in the greenhouse cage. Use of models allowed us to manipulate visual cues while keeping odor constant. Data were collected under, conditions similar to those of the previous assay, from July to November 1995. A model consisted of a painted plastic sphere, 38 mm diam. (Euromatic County, Leominster, Massachusetts, USA), with ten 3-mm holes punched in it, with an infested fruit inside as an odor source. To make walnut models that matched the color patterns of walnuts that wasps would encounter in the field, we used paint (Behr Premium Plus exterior acrylic latex flat) that was computer color-matched to a ripe fruit (green models) (Henneman and Papaj, 1999). We also painted models to represent fruits that had been damaged by feeding fly larvae, using similarly computer-matched yellow paint (Weatherfighter exterior acrylic latex flat), and brown paint (ProArt Liquid Tempera) (yellow/brown models) (Henneman and Papaj, 1999). Three green models and 3 yellow/brown models (all containing real, infested fruits) were made available to wasps in the cage. All wasps in this experiment had been exposed previously to infested fruits, which had been found to increase their landing response in the greenhouse (M. L. Henneman, unpublished data). Wasps were individually marked and released, and, as in the previous experiment, landings and time spent on each model were recorded for each individual.

Comparisons for all free-foraging behavioral assays were made using a randomization test for matched pairs, which is more powerful than conventional nonparametric methods (Edgington, 1980; Daniel, 1990). The matched pairs consisted of the number of landings by one wasp on one type of fruit or model versus the other type. The probability that each matched pair is random is calculated by comparing each actual pair to a distribution of 20,000 random pairs drawn from the actual data. Comparisons were run using SAS (SAS Institute, 1990).

Wind-Tunnel Assays. We tested the response of wasps to fruits both with and without visible damage. The wind tunnel measured 60 × 60 cm in cross section, and 180 cm in length. Airflow was 20 cm/sec, obtained by placing the downwind end of the tunnel in a fume hood. Illumination was provided by two 150-W incandescent floodlights, with white paper covering the roof of the tunnel in order to diffuse the incoming light. A patterned cloth was placed on the floor to provide an optomotor reference to flying wasps.

We tested wasps 11–25 d old that had emerged in the laboratory and had been exposed to infested walnuts for 2 hr 4–5 d previous to the test. A wasp was released from a small test tube on a platform at the same height at which a single row of walnut fruits was suspended, 20 cm from the floor of the tunnel. If the wasp flew to the side or ceiling of the wind tunnel, she was recaptured and released 1 or 2 more times. A choice was counted when the wasp landed on one of the fruits. Each wasp was tested once.

Three wind tunnel assays were conducted (Table 1). The first was similar to the free-foraging experiment using real fruit. Wasps were presented with an uninfested fruit, an infested fruit into which flies had oviposited 5 d previously (thus, fly larvae were present), and a damaged fruit from each of two walnut trees, for a total of 6 fruits. Damage was visible on the infested and damaged fruits. Positions of the fruit types were randomized every 7 landings by wasps.

The second assay was identical to the first except that the infested fruits had been oviposited into 1 d before, so that they contained fly eggs but no larvae, and lacked visible damage.

In the third assay, infested and uninfested fruits only were presented to wasps, also from two different trees. Fly oviposition in infested fruits occurred 3–4 d previously, and these contained larvae and some slight damage that was oriented upwind, and, thus, invisible to wasps.

TABLE 1. SUMMARY OF WIND-TUNNEL EXPERIMENTS[a]

Experiment	Walnut types (No. of choices) **U**	**I**	**D**	Days since fly ovip. in **I**	Damage visible?	Wasps tested (N)	Response rate (%)	χ^2 Test and P
1	1	9	9	5	yes	45	42.2	$\chi^2 = 6.737$; $P = 0.034$
2	0	0	0	1	no	22	0	
3	0	5		3–4	no	46	10.9	Binomial, $P = 0.03125$

[a] **U** = uninfested, **I** = infested, **D** = mechanically damaged. Two trees were represented in each treatment. **D** walnuts were damaged when physical damage appeared on **I**, at about 5 d after fly oviposition.

Walnut Fruit Volatile Analysis

Volatile Collections. Walnut scent was collected using dynamic headspace sorption (Raguso and Pellmyr, 1998). For each of the undamaged, damaged, and infested odor collections, 4 fruits were excised from 3 individual trees and enclosed in polyacetate oven bags (Reynolds, Inc.) cinched at 300-ml volumes with plastic ties. A electrical diaphragm vacuum pump (Manostat, Inc.) was used to pull scent-enriched air through glass cartridges packed with 30 mg of Tenax GC adsorbent (mesh size 20/35), at a flow rate of 250 ml air/min. Incoming air was drawn through glass cartridges packed with 50 mg of activated charcoal. Scent collections began at 10:00 AM and continued for 5 hr within a dimly lit laboratory, and blank collections were performed in parallel for each collection day in order to control for ambient contaminants. Headspace collections were repeated 5 times for the same fruits over a time course of 10 d so that we could follow time-related changes in the composition of volatile emissions. As damaged areas spread on infested fruits over time, we added mechanical damage to the damaged fruits. After collections, we weighed all walnuts and found no significant differences in fruit mass among the treatment groups (Kruskal-Wallis, $P > 0.3$ day 1–8, $P > 0.08$ day 10). Between collections, fruits were stored at 30°C within individual jars that contained wet sand to provide high humidity.

Sample Preparation and GC-MS Analysis. Trapped volatiles were eluted from adsorbent cartridges using 1 ml of HPLC grade hexane. Each sample was stored in 4-ml borosilicate glass vials with Teflon-lined caps at −20°C until analysis. Samples were concentrated to 75 μl using a flow of gaseous N_2, after which an internal standard of 3.5 ng toluene dissolved in 5 μl hexane was added to each sample to control for unequal concentration volumes (Raguso and Pellmyr, 1998) before injection. Volatile samples were analyzed using a Shimadzu GC-17A gas chromatogram (GC), combined with a Shimadzu QP5000 mass spectrometer (MS) as a detector. The MS was set at 1 scan/sec, scanning masses from 30 to 300 Da, with an EI detector voltage of 1.7–1.95 kV. MS parameters were adjusted daily correcting for baseline drift. Aliquots of 1 μl were injected into the GC (split-splitless injector, splitless mode, temp. 250°C), where component peaks were separated on a polar GC column (EC-Wax = Carbowax, 30 m length, 0.25 mm ID, 0.25 μm film thickness; Alltech, Inc.). Carrier gas was He with a flow rate of 1 ml/min and a split ratio of 1:2. After injection, the column temperature was held at 60°C for 3 min, increased at 10°C/min until 260°C, then held for 7 min. Samples were reinjected on a nonpolar GC column (EC-5; 30 m long × 0.32 mm ID, 1 μm film thickness) (see Levin et al., 2001 for temperature program) for independent evidence supporting compound identification.

Analysis of Chromatogram Data. Chromatogram peaks were tentatively identified through the use of online NIST and Wiley mass spectral libraries (over 120,000 spectra) and then by comparing their spectra and retention times on each

column with those of known standards. Volatiles also detected in ambient controls were subtracted from walnut chromatograms (see Pellmyr et al., 1987). The operational limit of detection using this protocol for total ion chromatogram MS mode is 14 pg of toluene.

Peak areas were integrated using Shimadzu QP5000 Class 5K software and quantitated via comparison with the internal standard. In order to determine whether gross differences in odor emission accompanied different treatments, we calculated total nanograms of scent per gram of fruit per hour, using the internal standard and fresh masses of the walnuts. We then performed repeated measures ANCOVA, using DAY as within-subject variable and TREATMENT as between-subject variable, with individual TREE as a covariate.

Volatile profiles were also compared across trees and treatments using principal component analysis of peak area data (Sokal and Rohlf, 1995). Principal component axes are calculated through hyperellipsoids formed by the correlation of all peak areas in multidimensional space. The first principal component axis (PC1) accounts for the greatest amount of variation in the data, and subsequent axes account for successively smaller amounts of variation. Peaks chosen for comparison were those that were resolved enough for their areas to be accurately calculated, and, through comparisons with other studies, were potentially relevant to herbivore-induced plant signaling and parasitoid host location (Paré and Tumlinson, 1997) (Appendix A). To examine changes in the profiles over time, the analysis was conducted on differences in peak areas between the first collection and each of four subsequent collections.

RESULTS

Greenhouse Cage Assay—Real Fruit. Wasps did not distinguish infested from damaged (mechanically damaged, uninfested) fruits in their landing behavior (Figure 1: Fisher's randomization test for matched pairs, $N = 15$, $P > 0.52$). In the second test, wasps landed more often on damaged walnuts than they did on uninfested (undamaged) walnuts (Figure 1: $N = 15$, $P < 0.01$). Wasps in the third test landed more often on infested walnuts than they did on uninfested walnuts (Figure 1: $N = 15$, $P < 0.021$).

Greenhouse Cage Assay—Artificial Models. Wasps did not land significantly more often on damaged models than on models resembling uninfested fruits (Fisher's randomization test for matched pairs, $N = 17$, $P < 0.09$). However, they approached models closely without landing at times, and if these approaches are added to the analysis, the effect of visual models is significant ($N = 17$, $P < 0.045$). Glas and Vet (1983) found a similar effect with the response of *Diachasmimorpha alloeum* to artificial fruit models, and suggested that, while visual models may be sufficient to attract *D. alloeum*, other cues may help them decide whether or not to land on a fruit.

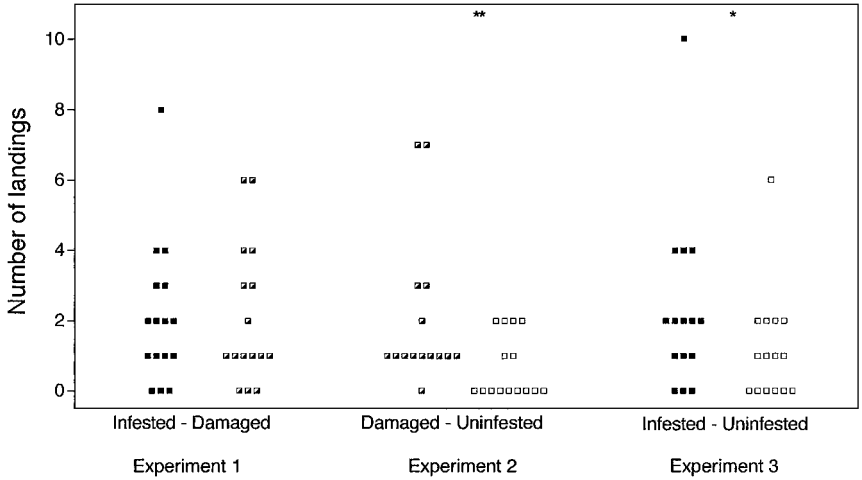


FIG. 1. Summary of all landings made by wasps in all three experiments in the free-foraging greenhouse assay using real uninfested, infested, and damaged fruits. Each square represents the total number of landings by a single wasp on one fruit type. In each experiment, there are totals for both types of fruit for each wasp. Open squares represent uninfested fruits, half-filled squares represent damaged fruits, and filled squares represent infested fruits. $^{*}P < 0.02$, $^{**}P < 0.01$ (Fisher's randomization test for matched pairs; see text).

Wind-Tunnel Assays. Forty-five wasps were released in the first experiment. The results were consistent with those of the free foraging assay (Table 1): more wasps landed on infested and damaged fruits than on uninfested fruits ($\chi^2 = 6.737$, $N = 19$, $df = 2$, $P = 0.034$). Of 22 wasps released in the second assay, in which eggs but no larvae were present in infested fruits, none landed on any fruits (Table 1). Finally, 46 wasps were released in the third experiment, of which all 5 that made a choice landed on infested fruits (Table 1: binomial test, $P < 0.001$).

Volatile Collections

Odor Emission. Overall changes in total volatile emission did not differ dramatically among infested, damaged, and uninfested control walnuts (Table 2), except for an unusual depression of volatile emission among all first-day manually damaged walnuts (Figure 2). Instead, odor differences associated with treatment were subtle and time-dependent (see below). The only significant differences in total odor emission found were among days, which is largely due to odor emission decreasing over time in all treatments (Figure 2), and DAY $\times$ TREE interaction, probably due to genetic variation among individual study trees (Table 2).

TABLE 2. REPEATED-MEASURES ANCOVA ON TOTAL ODOR EMISSION OVER 10-DAY PERIOD

Factor	*SS*	*df*	*MS*	*F*	*P*
Within subjects					
DAY	0.0169	4	0.0042	7.771	0.001
DAY × TREE	0.0099	4	0.0025	4.558	0.009
DAY × TREATMENT	0.0079	8	0.0010	1.820	0.132
Error	0.0109	20	0.0005		
Between subjects					
TREE	0.00005	1	0.00005	0.036	0.858
TREATMENT	0.0011	2	0.0006	0.423	0.676
Error	0.0066	5	0.0013		

Compound Identifications. All compounds identified in the walnut volatile analyses are listed in Appendix A. Volatile chemistry was overwhelmingly isoprenoid; 38 of the 81 compounds were monoterpene-related, while an additional 34 were sesquiterpene hydrocarbons or alcohols. Most remaining compounds were aliphatic alcohols, ketones, or acetates, some of which are lipoxygenase-derived and associated with general plant wounding (Paré and Tumlinson, 1997, 1999).

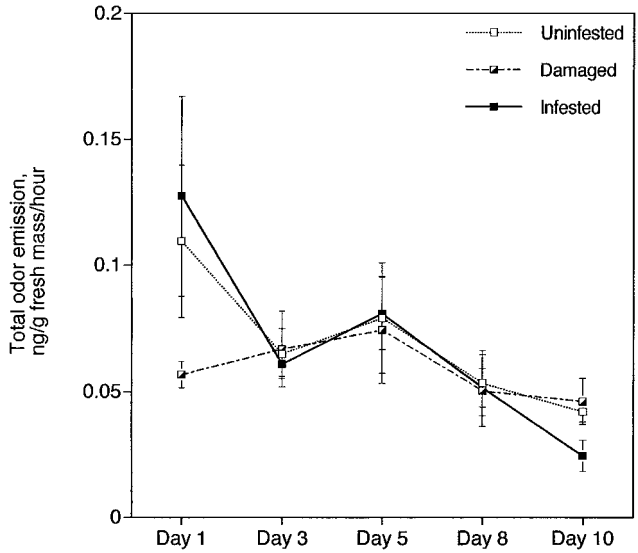


FIG. 2. Plot of total volatile emission for all treatments over the course of the study. Error bars represent standard error.

TABLE 3. RETENTION TIMES AND IDENTITIES OF COMPOUNDS USED IN PRINCIPAL COMPONENTS ANALYSIS[a]

Peak	Compound	Retention time, EC-wax (min)
2	**β-pinene**	4.00
3	**sabinene**	4.28
4	**myrcene**	5.16
5	**α-terpinene**	5.46
6	**2-methyl pentyl ketone**	5.54
7	**limonene**	5.82
8	**β-phellandrene**	6.00
9	**(Z)-β-ocimene**	6.50
10	γ-terpinene	6.69
11	**(E)-β-ocimene**	6.83
12	***p*-cymene**	7.15
13	**α-terpinolene**	7.35
14	(*E*)-**4,8-dimethyl-1,3,7-nonatriene**	7.75
15	3-methyl pentanol	8.02
17	**(Z)-3-hexen-1- ol**	8.91
20	6-hepten-2,4-diol	9.64
21	5-hydroxy-4-octanone	9.74
26	**α-copaene**	10.55
36	**β-caryophyllene**	11.98
37	**β-farnesene isomer**	12.74
45	**α-humulene**	12.88
74	**caryophyllene oxide**	16.39

[a] Identities of bold-faced compounds were confirmed from standard retention times.

Four compounds were found only in infested walnuts, in trace amounts: (*Z*)-3-hexen-1-ol and the putatively identified 3-methyl pentanol, 6-hepten-2,4-diol, and 5-hydroxy-4-octanone. Other compounds [isomers of bergamotene and hexenyl butyrate, indole, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, methyl salicylate] characteristic of herbivore-induced emissions in cotton, maize, soy, and tobacco (Turlings and Tumlinson, 1992; Dicke, 1994; Röse et al., 1996; McAuslane and Alborn, 1998; Halitschke et al., 2000) were not detected in any walnut samples. Aromadendrene was absent from tree 1, α-guaiene was absent from tree 2, and four other compounds [terpinene-4-ol, β-farnesene isomer 1, (*Z*)- and (*E*)-nerolidol] were absent from tree 3. γ-Gurjunene was absent from both trees 1 and 2. Identifications of the peaks chosen for comparative multivariate analysis of fruit types are presented in Table 3.

Profile Analysis. There were no consistent qualitative differences among uninfested, damaged, and infested fruits at any point in the time series, but there was

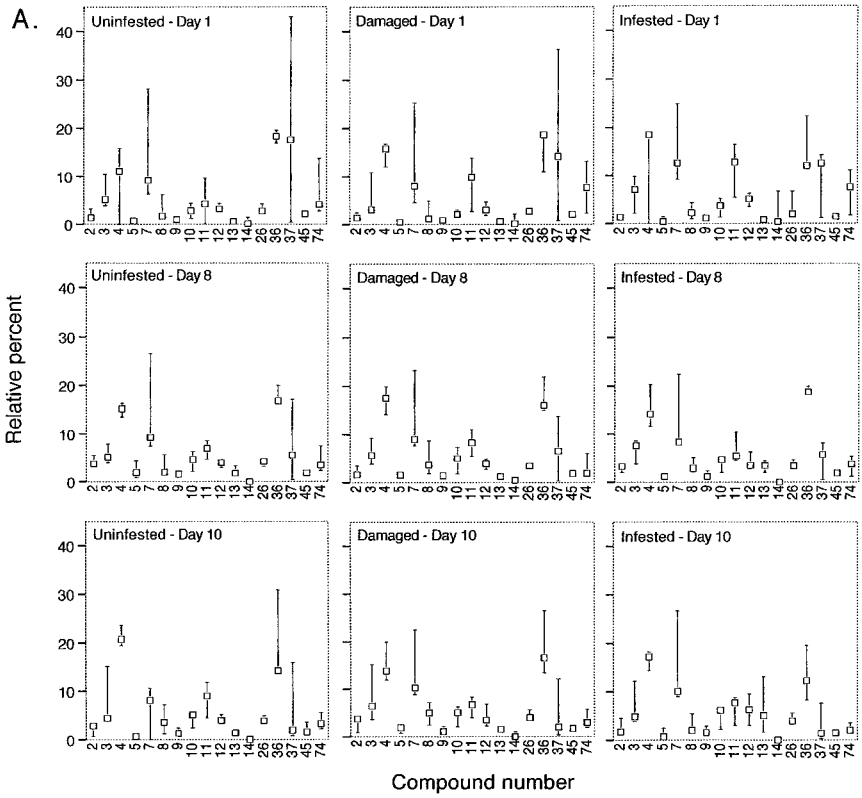


FIG. 3A. Relative peak areas for volatile collections from uninfested, damaged and infested fruits, from all three trees. Plots within A and B are all at the same scale, and show changes in relative peak areas from day 1 to day 8, to day 10. Labels on the *x* axis correspond to compounds listed in Table 2. Each square, low bar and high bar represents a peak area for one tree, divided by the total area of all the peaks in that trace for that tree, $\times 100$. Terpenoids are represented by hollow squares in A; aliphatic ketones and alcohols, compounds 6, 15, 17, 20, and 21, are represented by filled squares in B.

a quantitative effect of an increase in the amounts of aliphatic alcohols and ketones relative to terpenoids in infested fruits in later stages of infestation (Figure 3B). The first volatile collections (when only eggs were present in the infested walnuts) were similar across treatments, but by day 8 clear quantitative differences emerged among the treatments. Infested walnut fruits emitted greater amounts of aliphatic compounds relative to terpenoids than did damaged fruits, whose emissions were greater than uninfested fruits.

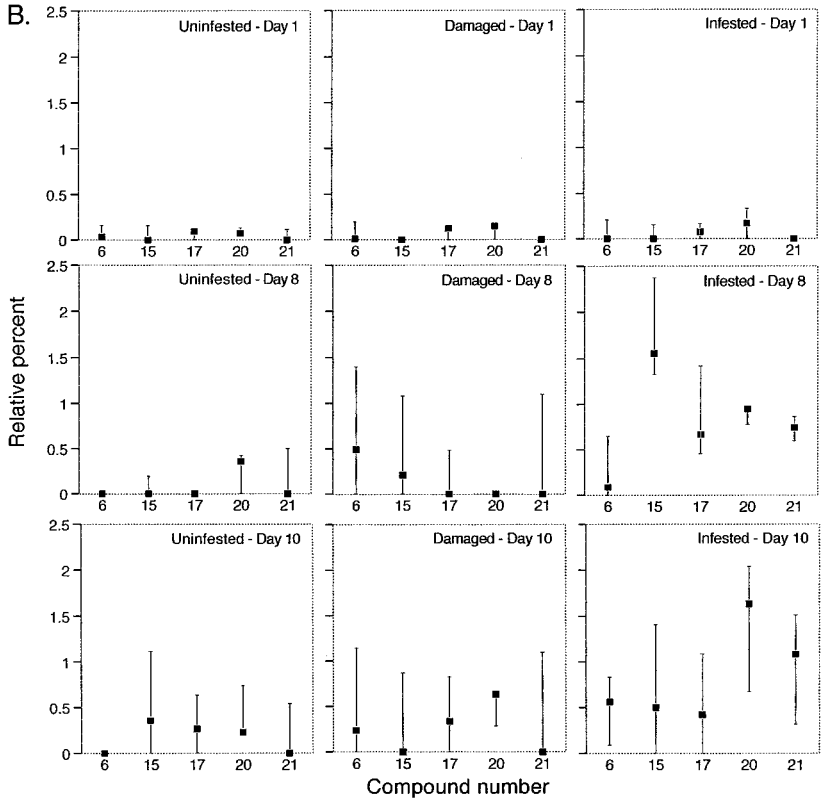


FIG. 3B. Continued.

These differences are quantified in Figure 4A, which plots principal component 1 (PC1) and principal component 2 (PC2) (accounting for 68% and 19% of the variation in the data, respectively) for each TREATMENT × TREE over time. Each symbol on the graph represents a decay profile, or change in the profile compared to day 1. Symbols that are closer together on the graph have similar decay profiles. Decay profiles of all uninfested and damaged fruits, clustered together, were similar throughout the experiment, especially along the PC2 axis. In contrast, the decay profile for the infested fruits from tree 1 on day 3 (when fly eggs had begun to hatch) differed from the others along PC2. On day 5, the decay profiles of all infested fruits were different from the main group, and on day 8, which corresponded to 80% visible damage on infested fruits, these differences were especially pronounced. By day 10, when the infested fruits had turned completely black with feeding damage, two of the infested-fruit treatments had

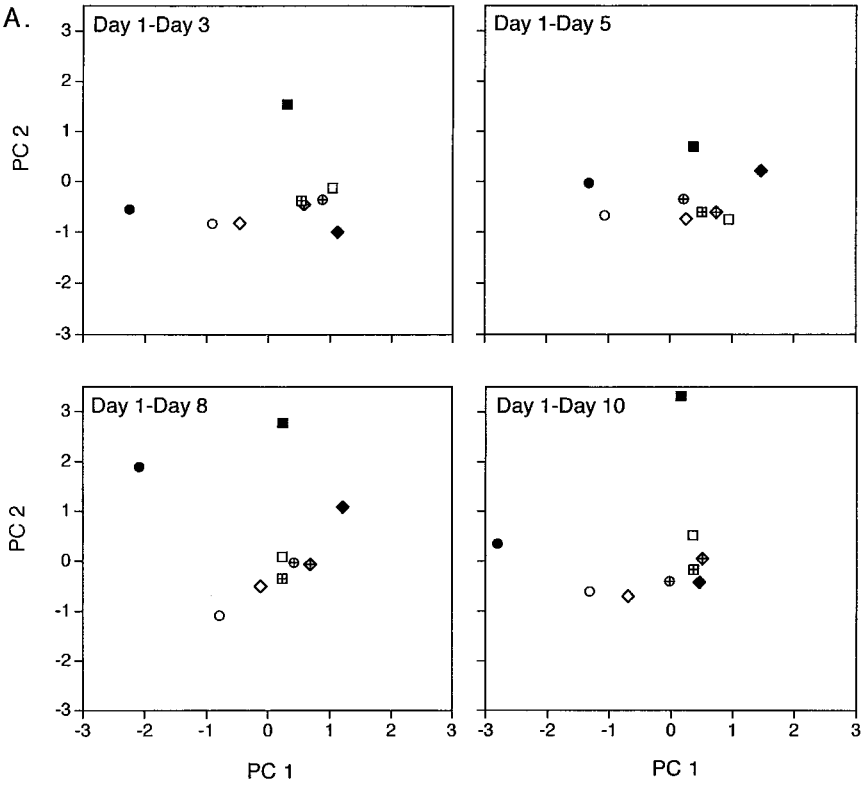


FIG. 4A. Plots of the first two principal components generated by the analysis. The four plots track the changes in volatile compounds over time, with the first collections subtracted from each subsequent collection (days 3, 5, 8, and 10). PC1 accounts for 68% of the variation in the data, and PC2 accounts for 19% of the variation. Symbols of the same shape represent fruits from the same tree: tree 1 = square, tree 2 = circle, tree 3 = diamond. Open symbols represent uninfested fruits, hatched symbols represent damaged fruits, and filled symbols represent infested fruits. (B) Vector correlation plot of peak loadings, showing PC1 (x axis) plotted against PC2 (y axis) (both ranging from -1 to 1) for each compound, with vectors originating from (0,0). Numbers on the vectors correspond to peak numbers listed in Table 3 and Appendix A and vector correlation values are given in Appendix A. Longer vectors point to compounds which influence more the location of the points in A. Solid vectors point to terpenoids, most of which influence mainly PC1; dashed vectors point to aliphatic ketones and alcohols, which influence mainly PC2.

decay profiles along PC2 similar to the main group of uninfested and damaged fruits.

The effects of aliphatic ketones and alcohols are suggested by a vector correlation plot (Figure 4B). For example, differences in profiles between infested fruits

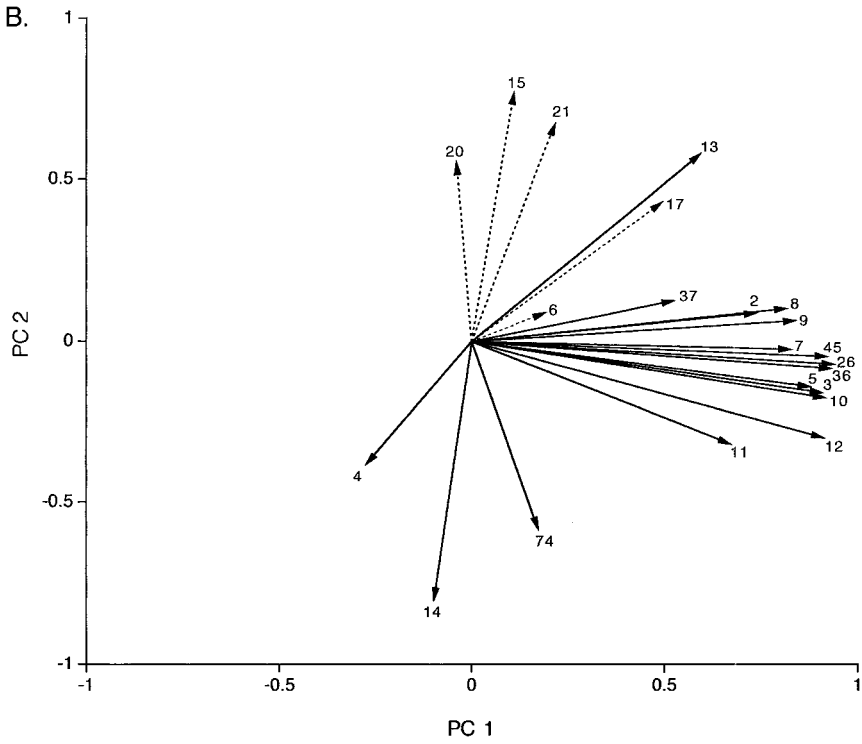


FIG. 4B. Continued.

and the other treatments in Figure 4A can be attributed to the effects of compounds strongly positive for PC2 in Figure 4B—tentatively identified as 3-methyl pentanol (compound 15), (*Z*)-3-hexen-1-ol (17), 6-hepten-2,4-diol (20), and 5-hydroxy-4-octanone (21). In contrast, differences among terpenoids—particularly limonene (7), copaene (26), caryophyllene (36), and humulene (45)—primarily account for the separation of individual trees in Figure 4A along PC1, even though some of these compounds are typically associated with constitutive plant responses to mechanical wounding (Paré and Tumlinson, 1997).

Contrary to previous results that showed the induction of β-farnesene (compound 37, Figure 3A) from living leaves infested by herbivores (Röse et al., 1996), we found no increase in this compound due to infestation. Also contrary to expectations, the emission rate of homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene, a universal herbivore-induced volatile signal in foliage, decreased relative to that of the aliphatic ketones and alcohols (compound 14, Figure 4B). A similar pattern was observed for caryophyllene oxide (compound 74, Figure 4B),

and myrcene (compound 4, Figure 4B). Finally, compounds such as β-pinene (compound 2), caryophyllene (compound 36), and humulene (compound 45), did not show damage-specific increases when compared with undamaged walnuts (Figure 3A).

DISCUSSION

Diachasmimorpha juglandis females distinguish host-infested from uninfested walnut fruits before alighting (Henneman, 1996, 1998). The results of the greenhouse free-foraging assays indicate that wasps use the presence of damage on a fruit as a cue for the presence of hosts because they did not distinguish in their landing choices between infested and mechanically damaged walnut fruits but did prefer damaged to uninfested fruits. Their failure to distinguish between infested and mechanically damaged fruits is intriguing. A large body of previous research indicates that many species of parasitic wasps prefer odors from plants damaged by hosts to qualitatively distinct odors emitted by mechanically damaged plants (e.g., Turlings et al., 1990, 1991a; Whitman and Nordlund, 1994); however, there are some other known exceptions, including a congener of *D. juglandis* that also attacks tephritids in fruits. *D. longicaudata* is not attracted to uninfested peaches but was found to be attracted equally to peaches infested with Caribbean fruit fly larvae and uninfested, but rotting, peaches (Greany et al., 1977). A similar response was found in *Campoletis sonorensis*, an ichneumonid that attacks noctuid moth larvae on many species of plants (McAuslane et al., 1991). A lack of discrimination between mechanical damage and insect-specific damage might be expected whenever plant damage is reliably caused by a host (Vet et al., 1991; Vet and Dicke, 1992). This is almost certainly the case for *D. juglandis*, a specialist on walnut flies, which are the dominant insect frugivores on walnuts. It is probably the case for *D. longicaudata*, which in Hawaii attacks several tephritids in several species of fruit (Leyva et al., 1991). *C. sonorensis* is an extreme generalist, attacking at least 27 species of noctuids (McAuslane et al., 1991), implying that several types of damage would be caused by an acceptable host.

Diachasmimorpha juglandis is apparently sensitive to olfactory differences between uninfested and infested fruits in the early stages of infestation; wasps distinguished fruits in the wind tunnel only 3–4 d after oviposition by flies, when eggs are just beginning to hatch, and odor profiles in infested fruits are just beginning to diverge from those of uninfested fruits (Figure 4A). This suggests that subtle olfactory cues alone are sufficient to attract foraging wasps. In addition, the artificial-model assay indicated that wasps can also use visual cues alone to distinguish between infested and uninfested fruits. The combination of all wind-tunnel results strongly suggests that visual damage is important for attracting wasps, in spite of

the presence of odor: the response rate in the wind tunnel increased more than fourfold when visual cues were available (Table 1).

Parasitoids are well known to use plant odors to locate their hosts (e.g., McAuslane et al., 1991; Kester and Barbosa, 1992; Poolman Simons et al., 1992; Vet and Dicke, 1992; Turlings et al., 1993b). The use of vision is less well known. In a field cage/sticky-trap assay, *D. longicaudata* used color to make landing decisions only in the absence of odor (Messing and Jang, 1992). However, Wäckers (1994) found that olfactory and visual cues had additive effects on the ability of the braconid *Microplitis croceipes* to find leaf-feeding host larvae (*Helicoverpa* spp.). Using both types of cues together, wasps have more complete information and are more successful in finding infested fruits (Drost and Cardé, 1992; Wäckers and Lewis, 1994). Messing and Jang (1992) found that *D. longicaudata* females are able to use odor to orient to fruits over several meters under field conditions. However, they were choosing among different species of fruits rather than infestation levels of the same species. In the canopy of a walnut tree interspersed with infested and uninfested walnuts, visual cues may be more helpful than odors to parasitoids orienting to specific fruits from distances of $\geq$1 m. After they approach a fruit, *D. juglandis* characteristically hover close to the fruit surface for up to 1 sec before they either alight or back away and fly off in another direction. At this stage, wasps may be assessing volatiles in order to decide whether to land (Glas and Vet, 1983). Visual cues have also been found to attract *D. longicaudata* in a wind tunnel (Jang et al., 2000).

Walnut volatiles were collected in order to describe differences among profiles of uninfested, artificially damaged, and infested fruits over time. Overall odor emissions were similar for all treatments. Although total volatile emissions were lower in damaged walnuts than in undamaged or infested walnuts on day 1, independent of tree, damaged fruits were in the same physical state as uninfested fruits at this time, so this difference is not biologically significant. The interaction of DAY $\times$ TREE (Table 2) indicates that trees changed in their total odor output differently over time. There were some qualitative chemical differences among trees; a few terpenoid compounds did not occur in all three trees (Appendix A). Furthermore, quantitative amounts of compounds present in all trees ranged widely (Figure 3). These trees were located at the same field site but differed in other consistent ways, such as timing of fruit development. Genetic differences may account for ecological as well as chemical variation. Because of these differences, replication was a useful, and necessary, approach to the identification of biologically significant differences among treatments. Our data also show some interesting variation in odors based on walnut phenological stage and infestation status. Volatile profiles in numerous fruits, including citrus and soursop (*Annona muricata*), change over time as they become ripe (Kekelidze et al., 1989; Iwaoka et al., 1993) and in apples as they become infested with codling moth larvae (Hern and Dorn, 2001).

Many of the compounds identified from the walnut volatile samples are common mono- and sesquiterpenes. Myrcene, limonene, copaene, and caryophyllene

are present in such taxonomically distinct plants as pine (Barnola et al., 1994) and cotton (Loughrin et al., 1994). Although foliage volatiles are most often studied, limonene, caryophyllene, and other terpenes also occur in citrus fruits (Kekelidze et al., 1989), linalool occurs in apricot fruits (Chassagne and Crouzet, 1995), and α-copaene has been found in tomato (Buttery and Ling, 1993). Mango contains α-pinene, α-terpinolene, *p*-cymene, and limonene, the latter two of which are attractive to the medfly (Hernandez-Sanchez et al., 2001). In addition to attractants to both herbivores (Pivnick et al., 1994) and their natural enemies (Turlings et al., 1993a; Dicke, 1994), terpenes are known to act as feeding repellents (Zhang and States, 1991; Barnola et al., 1994) and, in the context of induced wound responses, as oviposition deterrents (Kessler and Baldwin, 2001). Our data suggest that volatile terpenes are attractive to *D. juglandis*.

These previous studies and the present one suggest that novel compounds are not induced in fruit as they can be in leaf material (e.g., Turlings et al., 1990; Dicke and Van Loon, 2000). Larval infestation of walnut fruits appears simply to accelerate the rotting process, rather than induce novel volatile odors as found in other systems. The most obvious change over time in infested fruits was the increase in production of ketones, which began to occur in uninfested and damaged fruits in the latter stages of the experiment. Figure 3B shows that in uninfested and damaged treatments, compounds 6, 15, 17, 20, and 21 increased very slightly, but they increased more quickly in infested fruits. The one slight difference between uninfested and damaged walnuts was in compound 6, 2-methyl pentyl ketone, which appeared after physical and herbivore damage, but not in uninfested, undamaged fruits.

According to the analysis, the production of aliphatic alcohols and ketones was at least partly responsible for the separation of the infested fruits from the other treatments by day 8 (Figures 4A and B). The largest consistent separation in change between infested fruits and the other treatments occurred at day 8. By day 10, most infested walnuts were completely black and relatively odorless (Figure 2). Interestingly, when walnut fruits are black, they are less attractive to parasitoids (Henneman, 1998). Overall, we conclude that the profiles of mechanically damaged walnuts changed at a rate more similar to those of uninfested walnuts than to those of infested walnuts. This, combined with wind-tunnel results, suggests that wasps provided with visual information were ignoring olfactory information.

In summary, this study indicates that *D. juglandis* females foraging for hosts choose walnuts based on the presence of damage rather than the presence of host larvae *per se*. This lack of distinction makes sense in a system in which damage is nearly always caused by a host. Most wasps distinguished infested walnuts from uninfested walnuts using only olfactory information and distinguished simulated-damaged plastic walnut models from simulated-uninfested models using only visual information, suggesting that wasps can use either type of cue in isolation if necessary. The volatile profiles together with the greenhouse

behavioral assay using real walnut fruits suggest that at a distance, wasps tend to ignore odor and rely on visual cues, perhaps because in nature, odor plumes are indistinct and difficult for a wasp to follow until it is close to the fruit.

APPENDIX. VOLATILE COMPOUNDS IDENTIFIED FROM WALNUT HEADSPACE VIA GC-MS[a]

Compound	Retention time (min)		Relative abundance (%) (calculated on day 1), mean ± SE ($N = 9$)	PCA vector correlations	
	EC-wax	EC-5		PC1	PC2
1. α-pinene	2.54	8.51	0.69 ± 0.16		
2. β-pinene	4.00	9.32	1.05 ± 0.17	0.76779	0.08114
3. sabinene	4.28	10.22	4.72 ± 1.11	0.92456	−0.15096
4. myrcene	5.16	10.40	13.53 ± 2.56	−0.28265	−0.36069
5. α-terpinene	5.46	11.00	0.50 ± 0.16	0.89126	−0.15375
6. 2-methyl pentyl ketone	5.54	8.46	< 0.05	0.19985	0.0798
7. limonene	5.82	11.23	9.42 ± 1.76	0.86146	−0.02216
8. β-phellandrene	6.00	10.78	1.66 ± 0.41	0.84954	0.09719
9. (Z)-β-ocimene	6.50	11.26	0.70 ± 0.13	0.87258	0.0616
10. γ-terpinene	6.69	11.72	2.26 ± 0.59	0.94795	−0.15553
11. (E)-β-ocimene	6.83	11.40	8.33 ± 2.11	0.69905	−0.30329
12. *p*-cymene	7.15	11.13	2.89 ± 0.50	0.93062	−0.27905
13. α-terpinolene	7.35	12.26	0.55 ± 0.09	0.61612	0.54661
14. (*E*)-4,8-dimethyl-1,3,7-nonatriene	7.75	12.57	0.81 ± 0.48	−0.09455	−0.75629
15. 3-methyl pentanol	8.02	9.92	< 0.05[b]	0.11874	0.72712
16. (*Z*)-3-hexenyl acetate	8.04	10.58	0.11 ± 0.02		
17. (*Z*)-3-hexen-1-ol	8.91	8.54	< 0.05[b]	0.5133	0.40881
18. 2,3,6 trimethyl–1,5 heptadiene	9.27	11.52	0.09 ± 0.01		
19. 5(methylene-cyclopropyl)-1,5 heptadiene 1-pentanol	9.37	12.99	0.13 ± 0.01		
20. 6-hepten-2,4-diol	9.64	4.88	< 0.05[b]	−0.03587	0.52097
21. 5-hydroxy-4-octanone	9.74	6.59	< 0.05[b]	0.22506	0.63726
22. dehydro-p-cymene	9.79	10.91	0.13 ± 0.01		
23. α-cubebene	10.06	16.45	0.05 ± 0.01		
24. (Z)-β-terpineol	10.11	11.90	0.21 ± 0.05		
25. 5(methylene-cyclopropyl)-1-pentanal	10.47	12.20	0.09 ± 0.01		
26. α-copaene	10.55	16.93	2.57 ± 0.71	0.95503	−0.05904
27. bourbonene	10.93	17.10	0.30 ± 0.15		

APPENDIX. CONTINUED

28. linalool	11.24	12.30	0.21 ± 0.08		
29. methyl citronellate	11.47	14.81	1.19 ± 0.25		
30. 6,6 dimethyl 2-methylene bicyclo-[2.2.1] heptan-3-one	11.63	13.62	0.11 ± 0.02		
31. longifolene	11.67	17.07	0.14 ± 0.03		
32. isobornyl acetate	11.75	15.47	0.23 ± 0.02		
33. β-elemene	11.86	16.87	0.55 ± 0.12		
34. α-guaiene	11.90	17.70	0.06 ± 0.03[c]		
35. terpinene-4-ol	11.95	13.82	2.44 ± 1.04[d]		
36. β-caryophyllene	11.98	17.63	8.44 ± 1.59	0.96976	−0.06767
37. β-farnesene isomer	12.35	17.22, 17.35	0.11 ± 0.03[d]	0.55917	0.11743
38. myrtenal	12.39	14.18	0.12 ± 0.02		
39. sabina ketone	12.42	13.48	0.20 ± 0.03		
40. aromadendrene	12.52	17.83	0.05 ± 0.02[e]		
41. *E*-pinocarvyl acetate	12.60	15.86	0.15 ± 0.02		
42. citronellyl acetate	12.69	16.17	3.00 ± 0.45		
43. β-farnesene isomer	12.74	17.69	13.22 ± 4.66		
44. γ-gurjunene	12.81	18.28	< 0.05[f]		
45. α-humulene	12.88	18.09	1.36 ± 0.19	0.95084	−0.08146
46. (*Z*)-citral	13.00	14.72	0.14 ± 0.01		
47. α-cadinene	13.09	18.61	0.36 ± 0.06		
48. α-terpineol	13.12	13.97	0.19 ± 0.02		
49. sabinol	13.20	13.59	0.14 ± 0.01		
50. (*Z*)-2-methylene-3-[1-methylethenyl] cyclohexanyl acetate	13.29	15.38	0.20 ± 0.01		
51. germacrene D	13.36	18.43	1.98 ± 0.79		
52. β-farnesene isomer	13.44	18.15	1.73 ± 0.44		
53. (*E*)-citral	13.56	15.18	< 0.05		
54. carvyl acetate isomer	13.60	16.08	0.25 ± 0.04		
55. (*Z*)-carveol	13.64	14.39	0.31 ± 0.04		
56. (*E*, *E*)-α-farnesene	13.71	18.35	0.17 ± 0.02		
57. neryl acetate	13.81	16.34	0.51 ± 0.11		
58. citronellol	13.85	14.35	< 0.05		
59. δ-cadinene	13.88	18.84	1.95 ± 0.58		
60. γ-cadinene	13.93	18.55	0.07 ± 0.03		
61. β-sesquiphellandrene	13.99	18.68	0.09 ± 0.02		
62. geranyl acetate	14.09	16.60	0.14 ± 0.03		
63. solanone	14.14	ND	0.10 ± 0.02		

APPENDIX. CONTINUED

Compound	Retention time (min) EC-wax	Retention time (min) EC-5	Relative abundance (%) (calculated on day 1), mean ± SE ($N = 9$)	PCA vector correlations PC1	PCA vector correlations PC2
64. 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-[1-methylethyl] naphthalene	14.18	ND	< 0.05		
65. myrtenol	14.22	13.90	0.13 ± 0.02		
66. 1,4-p-mentha-dien-7-ol	14.29	14.11	0.11 ± 0.01		
67. carveol isomer	14.67	13.59	0.18 ± 0.01		
67. calamenene	14.75	18.87	0.17 ± 0.02		
68. cuminol	14.83	13.85	0.12 ± 0.01		
69. 4-isopropylidene-7-methyl-6-methylene-2-octenoic acid methyl ester	15.29	19.02	0.17 ± 0.03		
70. 2 phenyl ethanol	15.53	12.65	0.26 ± 0.04		
71. cadala-1(10),3,8-triene	15.67	19.18	0.08 ± 0.01		
72. α-bisabolene epoxide	15.75	19.41	0.53 ± 0.11		
73. veridiflorol	16.29	18.17	0.71 ± 0.12		
74. caryophyllene oxide	16.39	19.83	4.73 ± 1.03	0.17639	−0.55033
75. (*Z*)-nerolidol	16.56	19.12	0.05 ± 0.01[d]		
76. (*E*)-nerolidol	16.78	19.12	0.10 ± 0.04[d]		
77. 1,5,5,8-tetramethyl-12-oxabicyclo-[9.1.0]-dodeca-1,4-diene	16.97	20.14	0.37 ± 0.06		
78. patchoulane	19.61	20.42	0.05 ± 0.01		
79. 1,3,3-trimethyl-2-oxabicyclo-[2.2.2.]-octan-6-ol acetate	19.89	20.76	0.98 ± 0.29		
80. isoaromadendrene epoxide	19.95	19.63	0.05 ± 0.02		

[a] Identities of compounds in bold type were confirmed from standard retention times.
[b] Present only after day 2 in infested treatment.
[c] Absent from Tree 2.
[d] Absent from Tree 3.
[e] Absent from Tree 1.
[f] Present only in Tree 3.

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IMPACT OF POLYPHENOLS ON GROWTH OF THE AQUATIC HERBIVORE *Acentria ephemerella*

CLAUDIA CHOI,[1] CHRISTA BAREISS, OLIVER WALENCIAK, and ELISABETH M. GROSS*

Limnological Institute University of Konstanz 78457 Konstanz, Germany

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Abstract—Larvae of *Acentria ephemerella* live fully submerged, feeding on submersed aquatic angiosperms such as pondweeds (*Potamogeton* spp.) and *Myriophyllum spicatum.* Only the latter contains high concentrations of hydrolyzable tannins known to interfere with the growth of insect herbivores. We tested whether larvae grow faster on *Potamogeton perfoliatus* or *M. spicatum* and whether this is due to polyphenols in their food source. Larvae originating from the same egg clutch grew faster and larger on *P. perfoliatus* than on *M. spicatum*. The same growth response was observed with larvae that spent winter diapause on either *P. perfoliatus* or *M. spicatum*. These larvae were fed either with their host plant or the other macrophyte. No prior feeding effect was found, but growth of larvae reared on *M. spicatum* was less than when grown on *P. perfoliatus*. Larvae from another egg-clutch reared on *M. spicatum,* either from lake or cultivated in aquaria, exhibited reduced growth on the lake plants. *P. perfoliatus* contained less than 1% and *M. spicatum* (aquarium or field material) between 5 and 9% phenolic compounds. No differences in nitrogen content of leaves were found, but apical shoot sections of *M. spicatum* exhibited a significantly higher nitrogen content than *P. perfoliatus*. Our results indicate that hydrolyzable tannins are responsible for the reduced growth of *Acentria* when fed with *M. spicatum*.

Key Words—Chemical defense, gallotannin, ellagitannin, aquatic angiosperm, freshwater macrophytes, *Myriophyllum*, pyralid moth.

INTRODUCTION

Herbivory on freshwater aquatic angiosperms can be as intense as on terrestrial vegetation (Cyr and Pace, 1993). Biomass loss of macrophytes (Lodge et al.,

*To whom correspondence should be addressed. E-mail: Elisabeth.Gross@uni-konstanz.de
[1]Present address: BIOBASE GmbH, Halchtersche Str. 33, 38304 Wolfenbüttel, Germany.

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1998) and even changes in macrophyte community composition due to herbivory by both vertebrate (van Donk, 1998) and invertebrate (Johnson et al., 1998; Gross et al., 2001) herbivores have been reported. In the last decade, many studies have shown that chemical defenses of freshwater macrophytes are important, occurring as frequent as in marine or terrestrial macrophytes (Newman et al., 1996; Bolser et al., 1998; Wilson et al., 1999; Kubanek et al., 2000).

Phenolic compounds and especially tannins (polyphenols) are widely recognized as effective herbivore deterrents in terrestrial (Feeny, 1970; Hagermann and Butler, 1991) and marine systems (Steinberg and van Altena, 1992) but not in freshwater plants. Submerged aquatic angiosperms generally contain lower concentrations of phenolic compounds compared to emergent or floating leaved macrophytes (Gross, 1999; Smolders et al., 2000). One exception is *Myriophyllum spicatum* L. (Haloragaceae), which contains high concentrations of phenolics (Smolders et al., 2000), especially gallo- and ellagitannins (Gross et al., 1996; Gross, 1999). Hydrolyzable tannins are the dominant type of phenolics present in *M. spicatum* and other Haloragaceae (Saito et al., 1989; Gross et al., 1996; Nakai et al., 2000). Tellimagrandin II (1,2,3-tri-*O*-galloyl-6 (*S*)-hexahydroxydiphenoyl-ß-D-glucose), the major polyphenol present in *M. spicatum*, has strong algicidal and cyanobactericidal properties (Gross et al., 1996). Its concentration is highest in apical shoot sections where it reaches 1–5% of dry weight and accounts for up to 15% of all hydrolyzable tannins present in milfoil (Gross, 2000; Gross, unpublished results). In addition, condensed proanthocyanidins have been isolated from *M. brasiliense* and *M. spicatum* (Saito et al., 1989; Nakai et al., 2000).

Acentria ephemerella SCHIFFERMÜLLER & DENIS (Lepidoptera; named *Acentria* in the following) are important herbivores on *M. spicatum* (Johnson et al., 1998, Gross et al., 2001). Larvae of this aquatic pyralid moth feed exclusively on aquatic angiosperm tissue and avoid filamentous epiphytic algae (Johnson et al., 1998; Gross et al., 2001). *Acentria* feed on a variety of submerged macrophytes, especially pondweeds (*Potamogeton* spp.), *Ceratophyllum demersum* and *Elodea canadensis*, but avoid others, e.g., *Chara* spp. and *E. nuttallii* (Johnson et al., 1998; Gross and Johnson, unpublished results). Feeding on *M. spicatum* occurs predominantly on the apical shoot sections (Gross et al., 2001, 2002) despite the fact that the highest concentrations of hydrolyzable tannins are present in these plant parts (Gross, 2000). *Acentria* can fully develop on all above-mentioned host plants, but differences exist in the proportion of individuals reaching adulthood (Gross et al., 2001).

Phenolic compounds, especially polyphenols (hydrolyzable and condensed tannins), are well known to interfere with growth and development of many insect herbivores (Feeny, 1976; Hagerman and Butler, 1991). Reduced growth in insects has two major drawbacks. First, a smaller size of female pupae generally results in a lower number of eggs laid by the adult female and a decline in their overall fitness (Haukioja and Neuvonen, 1985; Kaitaniemi et al., 1998; Osier et al.,

2000; Tikkanen et al., 2000). Second, prolonged larval development can result in a higher predation mortality (Feeny, 1976; Benrey and Denno, 1997). However, plant polyphenols may also benefit insect herbivores. Higher tannin content in food plants of *Lymantria dispar* decreased the effectiveness of the bacterial pathogen *Bacillus thuringiensis* (Appel and Schultz, 1994).

We asked whether the polyphenol-rich milfoil would cause changes in growth of larvae similar to that observed in terrestrial lepidopteran larvae. The objectives of our study were to test whether the growth of *Acentria* larvae: (1) is influenced by polyphenol-containing freshwater angiosperms, (2) depends on the amount of polyphenols present in *M. spicatum*, and (3) is determined by prior feeding experiences.

METHODS AND MATERIALS

Plant Samples. Samples of various submerged macrophytes (Table 1) were collected in lakes of the Finger Lakes area of New York, USA, or in northern and southern Germany. The screening for phenolic content in different macrophytes used whole upper shoots (25-cm long). When comparing *P. perfoliatus* and *M. spicatum* from Lake Constance, we dissected the upper 25-cm shoot sections into apical shoot sections, leaves, and stems. Plants were shock-frozen in liquid nitrogen, lyophilized, and stored in the dark at room temperature for further analysis.

Analysis of Plant Chemistry. All analyses were performed with ultrapure water and with analytical-grade (p.a.) or HPLC-grade solvents. Plant material was extracted with 50% aqueous acetone (v/v, 10 mg/ml solvent) over 2 hr and stirred constantly. Solids were centrifuged and the supernatant used for the analysis of the total phenolic content. Phenolic content was measured as described previously (Gross et al., 1996). Extracts were used in concentrations yielding absorbances (A_{750nm}) between 0.1 and 0.5 AU. Calibration curves with tannic acid (Sigma T8406) were used to calculate total phenolic content as tannic acid equivalents (TAE). The PVPP (polyvinylpolypyrrolidone, Sigma P6755) assay was performed to distinguish phenolic compounds from other redox active compounds of non-phenolic origin (Loomis and Battaile, 1966; Gross et al., 1996).

The carbon and nitrogen content of plant samples was measured with an Elementar Analysator NCS 2500 (CE Instruments/Thermoquest) using atropine sulfate as a standard (58.8% C, 4.0% N, C:N molar ratio 17:1). The protein content of leaves was determined by multiplication of the percent nitrogen content by 6.25 (Feeny, 1970). Soluble protein content was not determined because both Lowry and Bradford assays are sensitive to soluble phenolic compounds, and do not provide an exact measurement of protein content in plants with high concentrations of phenolic compounds (Gross, unpublished results).

Growth Assays. We performed three different growth assays using either newly hatched larvae of *Acentria ephemerella* (experiments I and II) from egg

clutches found in summer 1999, or diapausing larvae of *Acentria* (experiment III) found in late September 2000 on senescent shoots of *M. spicatum* and *P. perfoliatus.* All larvae originated from Lower Lake Constance. Macrophytes used in the experiments were either collected weekly in the lake or cultured prior to the experiments for at least three months in aquaria under constant conditions ($18^{\circ}C \pm 1^{\circ}C$, 16L:8D photoperiod, 60 μmol PAR/m^2/sec). Head capsule width of larvae was measured with digital imaging at a mean precision of 1.5%.

Experiment I. To test whether polyphenol-rich *M. spicatum* affects the growth of *Acentria* larvae, we used two different treatments. Either newly hatched larvae were fed lake- or aquarium grown *M. spicatum* or *P. perfoliatus*. A clutch of 340 eggs found on *P. perfoliatus* in Lower Lake Constance on July 12, 1999, was used. Larvae that hatched during July 20–23 were randomly distributed to 12-well microtiter plates (50 larvae per treatment, maintained individually), and the remaining larvae were distributed equally to 100-ml glass jars with the respective food. Water and food were renewed twice a week. The head capsule width of the larvae was measured at intervals of 6 to 12 d. Until day 41, larvae that died were replaced with larvae of equal size from jars fed with the same host plant. The experiment ended at day 89.

Experiment II. In this experiment, we compared whether *Acentria* larvae differ in growth on *M. spicatum* derived from the lake or from aquaria. In Lower Lake Constance, on August 6, 1999, a second egg clutch was found on *P. perfoliatus.* The 370 eggs that hatched between August 10 and 13 were divided into two groups according to the procedure described in experiment I. The experiment ended on day 72 (October 21, 1999) because *M. spicatum* became senescent at this time.

Experiment III. This assay was designed to investigate whether previous food experience would influence the outcome of the growth assays with polyphenol-rich and polyphenol-free freshwater macrophytes. *Acentria* hibernates in the larval stage and bores holes for shelter in lower stem sections of tall growing macrophytes, such as *P. perfoliatus* or *M. spicatum* (Gross et al., 2002). Larvae were collected on September 27, 2000, in Lower Lake Constance, near the island of Reichenau, and stored at $4^{\circ}C$. In March 2001, working on ice, larvae were removed from the stems of macrophytes and freed from their hibernicula. They were placed individually in 30-mm-diam Petri dishes and fed with leaves of the macrophytes used in the different treatments submerged in 5 ml of tap water. Larvae that originated from *M. spicatum* were then fed with either *M. spicatum* or *P. perfoliatus* (MSMS or MSPF treatment). Larvae that came from *P. perfoliatus* plants were fed with either one of the two macrophytes (PFMS and PFPF treatment). In each treatment, 24 larvae were used. Larvae were reared for 53 d, and head capsule width was measured at the start of the experiment and then every 6–10 d.

Statistical Analysis. Data were analysed using the Student *t* test, one- or two-way ANOVA, and Tukey HSD *post-hoc* tests (Statistica 99 edition or SigmaStat, version 2.03).

RESULTS

Plant Chemistry. The phenolic content of aquatic angiosperms belonging to several families varied from < 1% to 15%, measured as tannic acid equivalents (TAE) and based on dry mass dm, (Table 1). Several host plants of *Acentria ephemerella*, such as *Ceratophyllum demersum, Elodea canadensis* and *Potamogeton perfoliatus*, had < 1% TAE in their tissue. All members of the Haloragaceae (*Myriophyllum* spp., *Proserpinaca* sp.) contained > 6.5% dm phenolic compounds, approximately one order of magnitude higher than all other species investigated.

We tested the nutritional quality of the plants used in the growth experiments during late summer 1999. A comparison of leaves from the upper 25-cm shoot sections of *P. perfoliatus* and *M. spicatum*, sampled during late summer of 1998 and 1999, demonstrated that in both years the latter had a significantly higher phenolic content than *P. perfoliatus* (Table 2, 9% vs. 1% TAE in both years, $P < 0.006$). More than 95% of all tannic acid equivalents in *M. spicatum* were phenolic compounds and precipitated with PVPP. Approximately 50% of the tannic acid equivalents in *P. perfoliatus* were of nonphenolic origin. *M. spicatum* plants kept for several months in aquaria exhibited a lower leaf phenolic content than those taken directly from the field (aquarium: $5.5 \pm 0.4\%$ TAE, mean $\pm$ 2 SEM, $N = 9$; lake, samples taken in summers 1998–2000 at peak biomass of

TABLE 1. COMPARISON OF TOTAL PHENOLIC CONTENT OF VARIOUS SUBMERGED MACROPHYTES[a]

Aquatic angiosperms	Tannic acid equivalents (mg/g dm)		Different locations
	Mean $\pm$ 1 SD	Range	
Haloragaceae			
Myriophyllum alterniflorum	79.8 ± 11.0	67.6–88.9	3
M. heterophyllum	66.7 ± 6.6	62.4–74.3	3
M. spicatum	83.2 ± 8.8	68.7–101.7	15
M. verticillatum	67.5	65.4–69.6	2
Proserpinaca palustris	148.6	148.6	1
Other families			
Ceratophyllum demersum	8.3 ± 1.8	6.5–10.1	3
Elodea canadensis	6.0 ± 1.3	4.9–7.0	3
Najas flexilis	3.6	3.6	1
Potamogeton perfoliatus	9.4 ± 4.5	4.5–12.1	3
Ranunculus trichophyllus	18.9 ± 1.7	16.7–21.0	3

[a] Only data from plants taken during maximum plant biomass at different sites/in different lakes were taken into account. Upper 25 cm of shoot sections were taken for the analysis. Data are presented as tannic acid equivalents based on plant dry mass. Data represent means, 1 SD, and range of samples taken in late summer from different locations.

TABLE 2. COMPARISON OF TOTAL PHENOLIC CONTENT OF SUBMERGED MACROPHYTES USED AS HOST PLANTS IN OUR GROWTH EXPERIMENTS[a]

	Tannic acid equivalents (mg/g dm)			
	M. spicatum	*P. perfoliatus*	*t*	*P*
1998	88.0 ± 16.0	11.6 ± 7.8	8.5	0.001
1999	94.6 ± 30.0	12.1 ± 7.7	5.3	0.006

[a] Only leaves from the upper 25 cm of shoot sections were taken for the analysis. *M. spicatum* and *P. perfoliatus* were collected in Lake Constance during the maximum growing period (July–September) of 1998 and 1999 ($N = 3$ each; mean ± 2 SEM).

M. spicatum: 9.0 ± 1.0% TAE, $N = 9$, Student's t test, $t = 6.0$, $P < 0.001$). Whereas the phenolic content of aquarium plants stayed more or less the same, we observed a steady decline in field milfoil from 12% at the beginning of August to 7% at the end of September and < 5% TAE at the end of October 1999 (linear regression, TAE = 333.5 − 0.99 × (time); $R = -0.97$; $P < 0.001$). When comparing the phenolic content of *M. spicatum* samples taken in late September to late October 1998–2000 with those of aquarium plants, no difference was found (*M. spicatum* fall 5.8 ± 1.3% TAE, $N = 6$; aquarium samples as above, $t = 0.5$, $P = 0.7$).

Nitrogen content is commonly used as a proxy for protein content and thus nutritional quality of host plants for herbivores. Leaves of *P. perfoliatus* and both field- or aquarium grown *M. spicatum* had a nitrogen content of 3.6–3.8% dm, and were not significantly different (Table 3; one-way ANOVA, $P = 0.8$). The apical shoot sections of *P. perfoliatus* exhibited a significantly lower nitrogen content (3.3%) than those of either aquarium or field *M. spicatum* (4.8–5.4%; Table 3; one-way ANOVA, $P < 0.001$). Using the conversion factor of 6.25 (Feeny, 1970), to estimate the content of soluble protein, we calculated a content of 22–24% in

TABLE 3. NITROGEN CONTENT (% OF DM) OF MACROPHYTES USED IN GROWTH EXPERIMENTS WITH *Acentria* LARVAE[a]

	Nitrogen					
	M. spicatum		*P. perfoliatus*			
	Field	Aquarium	Field	*MS*	*F*	*P*
Leaves	3.6 ± 0.4 a	3.6 ± 0.4 a	3.8 ± 0.3 a	0.1	0.3	0.8
Apical shoots	4.8 ± 0.4 a	5.4 ± 0.5 a	3.3 ± 0.3 b	8.6	20.1	<0.001

[a] Data represent means ± 2 SEM of 6 to 12 independent samples. Results of one-way ANOVAs for each plant part are presented. Different letters indicate treatments that vary significantly at $\alpha = 0.05$.

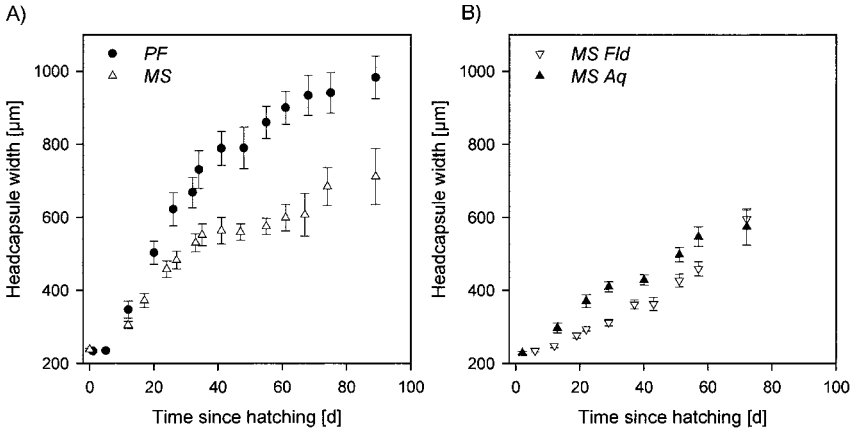


FIG. 1. Newly hatched *Acentria* larvae reared on different freshwater macrophytes. Growth was measured as headcapsule width. A) Experiment I: *Acentria* reared on *Potamogeton perfoliatus (PF)* and *Myriophyllum spicatum (MS)*. B) Experiment II: *Acentria* reared on fresh *M. spicatum* harvested weekly from the lake *(MS Fld)* or cultured in aquaria *(MS Aq)*. $N = 50$ each per treatment in both experiments, $N = 14$–15 per treatment at the end of the experiments. Data represent means $\pm$ 2 SEM.

leaves of *P. perfoliatus* and *M. spicatum*. The carbon content of all plants ranged between 35 and 42% dm and the C:N molar ratio varied from 8:1 to 14:1.

Growth Experiments. In experiment I, larvae developed differently on *P. perfoliatus* and *M. spicatum* (Fig. 1A). Larvae did not differ in size at the beginning of the experiment (*M. spicatum* head capsule widths $= 238 \pm 2\mu$m; *P. perfoliatus* $= 234 \pm 2\mu$m, mean $\pm$ 2 SEM, $N = 50$ each, $P = 1.0$). The average head capsule width of larvae reared on *P. perfoliatus* was larger than when fed with *M. spicatum* (Figure 1A). The two-way ANOVA showed a significant treatment effect ($F_{1,554} = 225.5$, $P < 0.001$), a significant time effect ($F_{7,554} = 180.2$, $P < 0.001$), and the interaction of treatment and time was also highly significant ($F_{7,554} = 9.6$, $P < 0.001$). From the third week until the end of the experiment, larvae were larger on *P. perfoliatus* than on *M. spicatum* ($P < 0.05$). The largest larvae on *P. perfoliatus* reached a head capsule width of 1190 μm; the average size being $983 \pm 58\mu$m ($N = 14$). Some larvae fed with *M. spicatum* also reached a head capsule width of more than 900 μm, but not until the end of the experiment; others stayed well below 600 μm, resulting in the lower mean size ($712 \pm 76\mu$m, $N = 15$). In both treatments, larval size reached a plateau after 40 days, indicating the end of growth.

Experiment II was performed with another egg clutch found on *P. perfoliatus*. This was divided into two treatment groups; one group was fed fresh *M. spicatum* collected weekly in the field, and one was fed *M. spicatum* from aquaria. During most of the experiment, larvae fed with aquarium plants grew better than those

reared on field *M. spicatum* (Figure 1B). Again, in the two-way ANOVA we observed a significant treatment ($F_{1,242} = 199.8$, $P < 0.001$), a significant time ($F_{4,241} = 51.0$, $P < 0.001$), and a significant interaction effect ($F_{4,241} = 5.0$, $P < 0.001$). *Post-hoc* tests revealed significant differences at days 13 to 57 ($P < 0.005$), but not at the end of the experiment (day 72, $P = 0.98$). At this time (end of October) we had to stop the experiment, because milfoil started to decay in the lake. During September and October 1999, field milfoil no longer had a higher phenolic content compared to plants from the aquaria (field 5.8%, aquarium 5.5% TAE, $t = 0.5$, $P = 0.7$).

In experiment III, we investigated whether previous feeding experience would influence the growth of *Acentria* on polyphenol-rich or polyphenol-free macrophytes. This experiment was conducted with larvae that had started winter diapause in either *P. perfoliatus* or *M. spicatum*, and we assumed that these were the plants the larvae fed on previously. Larvae dissected from *P. perfoliatus* or *M. spicatum* stems did not differ in size (mean head capsule width all larvae 556 $\pm$ 16 μm; mean $\pm$ 2 SEM; range 400–707 μm). Larvae then developed differently depending on the macrophyte being offered for food. The two-way ANOVA showed a significant treatment effect ($F_{3,362} = 18.8$, $P < 0.001$), a significant time effect ($F_{4,362} = 293.1$, $P < 0.001$) and a significant time $\times$ treatment interaction effect ($F_{12,362} = 6.9$, $P < 0.001$). Starting with day 15, larvae fed with *P. perfoliatus* (treatments PFPF and MSPF) were significantly larger than those shifted from *P. perfoliatus* to *M. spicatum* (PFMS) ($P = 0.015$). At the end of the experiment, larvae were either large (PFPF/MSPF), or small (PFMS/MSMS) with mean head capsule widths of 1014/989 or 891/834 μm, respectively (Figure 2). *Post-hoc* tests revealed that these treatments differed significantly at day 40 ($P < 0.02$) and day 53 ($P < 0.005$).

DISCUSSION

Our study shows that the performance of a true aquatic shredder–herbivore, *Acentria ephemerella*, is correlated with the presence and amount of phenolic compounds in their host plants. In our screening, all members of the Haloragaceae contained high concentrations of phenolic compounds, approximately one order of magnitude higher than other macrophytes investigated. Smolders et al. (2000) also found that *M. spicatum* had a high phenolic content, reporting a mean concentration in leaves of 8.4%. Phenolic compounds in this species are predominantly gallo- and ellagitannins (Gross et al., 1996). In contrast, according to our assays, *P. perfoliatus* always contained less than 1% phenolic compounds. In another study, *P. perfoliatus* contained less than 2% phenolic compounds (Smolders et al., 2000), and no tannins were found in this species (McClure, 1970).

The mean nitrogen content of 3.6–3.8% dm in leaves of the freshwater macrophytes used in our experiments fits well within the range found in other studies

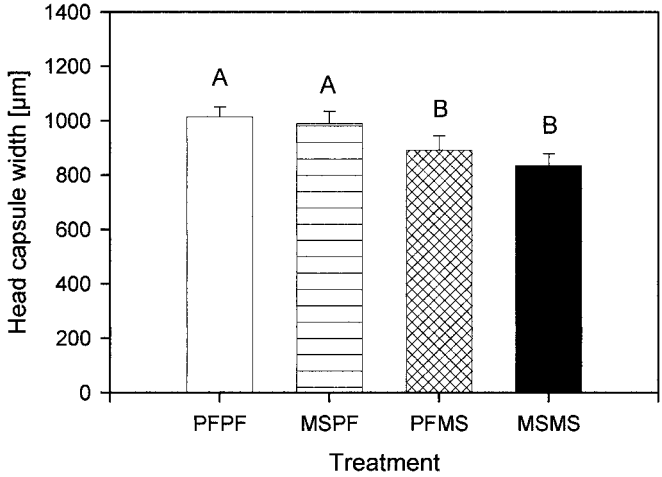


FIG. 2. Experiment III: final head capsule width of *Acentria* larvae that had a winter diapause in their life cycle in shoot sections of either *Potamogeton perfoliatus (PF)* or *Myriophyllum spicatum (MS)*. Larvae were fed in a reciprocal design with either the macrophyte they originated from or the other macrophyte (PFPF, PFMS; MSPF, MSMS). Data represent means $\pm$ 2 SEM of their size at day 53. Different letters indicate treatments that vary significantly at $\alpha = 0.05$.

(Nichols and Keeney, 1976; Spencer and Ksander, 1999). Nitrogen content was the same in all host plants, and was apparently not limiting the growth of *Acentria*. Therefore, the decreased size of *Acentria* in our experiments seems to be a result of the high concentration of polyphenols in *M. spicatum*. Two different experimental designs demonstrated that *Acentria* grew faster on *P. perfoliatus* than on *M. spicatum*. In experiments I and II, larvae differed significantly in size 3 wk after hatching, depending on the source of food offered. In these experiments, larvae originated from the same egg clutch. In experiment III, we used diapausing larvae retrieved from either milfoil or *P. perfoliatus*, suggesting they had used the respective plant for feeding prior to hibernation. Based on our laboratory findings, one would expect field larvae to be smaller on milfoil than on *P. perfoliatus* in fall. However, diapausing larvae from both macrophytes did not differ in size. This can be explained by examining the differences in life-cycle between the plants. Larvae first colonize *P. perfoliatus* in spring, and later move to *M. spicatum* at the end of summer (Gross et al., 2002). This is probably related to the earlier emergence of *P. perfoliatus* and later senescence of *M. spicatum* in Lake Constance. Thus, larvae on milfoil have a longer time for development and may reach the same size for hibernation as those already diapausing in lower stems of *P. perfoliatus* (Gross et al., 2002). Our results in experiment III indicate that larvae stay longer

at different instar stages with *M. spicatum*, thus reaching a similar size later than larvae fed with *P. perfoliatus*.

Apparently, not only the presence but also the amount of polyphenols present in *M. spicatum* determine the final size of *Acentria* larvae. *M. spicatum* from the field or from aquaria exhibited no difference in their nitrogen content. However, field plants contained, on average, twice as much polyphenols as aquarium plants. In the fall at the end of experiment II, this difference disappeared. At that time, larvae in both treatments were the same size. Unfortunately, we could not extend the experiment due to the senescence of *M. spicatum* in the lake. The final proof that polyphenols, especially hydrolyzable tannins, are solely responsible for the reduced growth of *Acentria* can only be obtained with a defined artificial diet to which selected polyphenols are added in various concentrations. Unfortunately, *Acentria* can not be reared on artifical diets. We have tested several commonly used lepidopteran diets (wheat germ-, pinto bean-based diets) or alginate- and agar-based diets used in marine studies. Larvae did not accept any of these and did not survive (Gross, unpublished results).

Feeding preferences of the semi-aquatic moth *Munroessa gyralis* (Pyralidae), were not related to phenolic compounds, protein concentration or the leaf toughness of macrophytes (Dorn et al., 2001). Field observations and laboratory tests show that *Acentria* larvae prefer the apical meristems of *M. spicatum* compared to lower shoot sections (Gross et al., 2001). Apical meristems contain significantly higher concentrations of polyphenols and tellimagrandin II than lower shoots (Gross, 2000). Despite the reduced growth observed in these experiments, larvae can fully metamorphose on *M. spicatum* (Johnson et al., 1998, 2000; Gross et al., 2001, 2002), and are frequently found associated with this macrophyte (Painter and McCabe, 1988; Newman and Maher, 1995; Johnson et al., 2000). Larvae may benefit from feeding at apical tissue of milfoil because of the higher nutrient content and a better protection from visually oriented predators (Gross et al., 2002). Additionally, the antimicrobial activity of polyphenols (Walenciak et al., 2002) may protect *Acentria* from bacterial and fungal pathogens and thus compensate for reduced growth. Whether milfoil polyphenols affect the overall fitness of *Acentria* remains to be elucidated.

In this study, we have shown that *Acentria* exhibits a reduced growth on the polyphenol containing *M. spicatum* compared to *P. perfoliatus.* This may ultimately lead to a lower female fecundity, as observed in many other lepidopteran studies (Haukioja and Neuvonen, 1985; Kaitaniemi et al., 1998; Tikkanen et al., 2000). The antinutritional effects of dietary tannins result most often in a depressed growth rate and feed utilization efficiency (Hagerman and Butler, 1991). Apparently, gut symbiontic microorganisms are also involved in the allelochemical interaction of polyphenols and herbivores (Walenciak et al., 2002). Nonetheless, the exact mechanism of tannin interference with digestion in Lepidoptera is still unknown.

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CHEMICAL MEDIATION OF EGG CAPSULE DEPOSITION BY MUD SNAILS

DAN RITTSCHOF,* PRASAD SAWARDECKER, and CAROLINE PETRY

Duke University Marine Laboratory Beaufort, North Carolina 28516, USA

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Abstract—Mud snails (*Ilyanassa obsoleta* = *Nassarius obsoletus* = *Nassa* obsoleta) deposit eggs in protective capsules on hard substrata in soft bottom environments. We studied sites of egg capsule deposition and snail movement responses to odors to determine if chemoreception plays a role in deposition site selection. From results of field surveys, laboratory experiments, and field experiments, we conclude that mud snails use chemoreception for capsule deposition. Attractive odors originate from mud snail and whelk egg capsules and from living bivalves. Evidence for attractive odors from conspecifics is equivocal. Capsules are deposited on living odor sources and nearby hard substrates. We hypothesize that deposition of capsules on living substrates increases the likelihood that embryos will survive by decreasing the chance of smothering of embryos by sediments.

Key Words—Reproduction, reproductive ecology, behavior, gastropods, egg capsule deposition, mud snail.

INTRODUCTION

Among aquatic gastropods, chemical cues, signals, and pheromones play roles in feeding (Carr, 1967a,b), prey detection, (Carriker, 1955; Rittschof et al., 1983), predator avoidance (Gore, 1966; Snyder, 1967; Atema and Burd, 1975; Duval et al., 1994; Rahman et al., 2000) alarm responses (Snyder, 1967; Atema and Burd, 1975; Duval et al., 1994; Rahman et al., 2000), species recognition (Gore, 1966; Atema and Stenzler, 1977; Stenzler and Atema, 1977; Bretz and Dimock, 1983; Hadfield and Pennington, 1990), predators (Dix and Hamilton, 1993; Duval et al., 1994; Rahman et al., 2000), and reproduction (cf: Painter, 1992; Painter et al., 1999). With respect to aggregation, breeding, and egg deposition, chemically mediated behavior of sea hares is a well-studied marine example.

* To whom correspondence should be addressed. E-mail: Ritt@duke.edu

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Sea hares, opistobranch gastropods, are relatively short lived simultaneous hermaphrodites that lay egg strings and mate in large aggregations during an extended reproductive season (Kupfermann and Carew, 1974; Audesirk, 1979; Susswein, et al., 1983, 1984). Temperature is a synchronizing cue (Smith and Carefoot, 1967, Audesirk, 1977). Egg string deposition occurs in specific habitats (Audesirk, 1979). Pheromones from egg-laying individuals and egg strings attract and stimulate other individuals to deposit eggs and to mate. Sperm are transferred in an immature state (Thompson and Bebbington, 1969), and mating does not cause ovulation (Blankenship et al., 1983). Egg laying hormone (ELH) initiates egg-laying and coordinates associated behaviors (Painter, 1992). A bout of egg laying releases pheromones that attract others and initiate mating (Painter, 1992). Attractive pheromones from the egg strings and egg-laying individuals maintain aggregations (Jahan-Parwar, 1976; Audesirk, 1979). At least one pheromone associated with egg strings may be a peptide from the ELH gene family.

The focus of our study is egg capsule deposition by mud snails (*Ilyanassa obsoleta* = *Nassarius obsoletus* = *Nassa obsoleta*). Mud snails are abundant intertidal prosobranch gastropods that occur in spectacular aggregations (Jenner, 1957) on soft bottom sediments on the Atlantic seaboard from Cape Cod to Florida. Mud snail responses to chemicals include scavenging (Dimon, 1905; Curtis, 1985), feeding (Carr, 1967a,b), trail following (Crisp, 1969; Trott and Dimock, 1978; Bretz and Dimock, 1983), alarm (Atema and Burd, 1975), imposex i.e., females with male secondary characters, (Smith, 1971, 1980; Oberdoerster et al., 1998), and alarm to predator odors (Rahman et al., 2000).

Mud snails breed on an incoming tide (Dimon, 1905). On the North Carolina, (USA) coast, breeding occurs in the fall and again in the spring during egg capsule deposition, February to May (Sastry, 1971). Egg capsule deposition is dependent upon water temperature (Scheltema, 1967). By the end of the spawning season, most surfaces where snails are breeding are coated with a continuous fuzzy layer of egg capsules (Jenner, 1957; Sastry, 1971). Our goals in this study were to demonstrate that snail aggregation and egg capsule deposition are chemically mediated and to elucidate sources of chemicals.

METHODS AND MATERIALS

Field Study Site. Field sites were the Carrot Island embayment, a 300-m × 600-m sand and mud flat in the Rachel Carson Estuarine Research Reserve (centered at 34°42.625 N, 76°40.230 W) and NOAA Beach, a 100-m × 40-m mud flat on the eastern side of Piver's Island (centered at 34°43.097 N, 76°40.267 W), in Beaufort, NC, USA. The Carrot Island Embayment was used as a field observation site. NOAA beach was the site of field experiments.

Egg Capsule Deposition. Based upon preliminary observations in 1991, we conducted four field censuses in 1992. We visited each census site only once and conducted each census in the 2 hr around the time of low tide. We first determined the frequency of capsule deposition on the first 120 shell substrates encountered while walking in mud snail areas. In a separate census, we determined the frequency of egg capsule deposition on the first 100 living and dead oysters encountered. In a third and fourth census, we determined the frequency of egg capsule deposition on the first 100 dead oyster valves encountered that were within 30 cm of a living oyster, and the first 100 dead oyster valves that were >30 cm from the nearest living oyster.

Sex, Seminal Receptacle Condition, and Parasitism in Snail Microhabitats. In 1991, we observed that mud snails occurred in tight (touching) aggregations on muddy substrates and around oysters. Snails on sand substrates were rarely touching. In 1992, we collected a total of 99 snails from 5 aggregations on mud, 99 snails from 5 aggregations around oysters, and 90 individuals from sandy areas. Snails were removed from their shells and their sex determined by the color of the gonad. Male gonads are bright orange and female gonads are cream colored (Sastry, 1971; Smith, 1980). Imposex rates in this area are 0–5% (Oberdoerster et al., 1998). We determined whether each female had a full (white) or empty (not white) seminal receptacle, and we determined if snails were parasitized. The gonad and digestive gland were broken open and examined with a dissecting microscope for the presence of life history stages of trematode parasites (Curtis, 1987).

Finally, we looked for pairs of snails in all three microhabitats. Pairs were picked up and pulled apart. If a penis was observed, the pair was counted as copulating, and each was placed in an individual container and returned to the lab for determination of sex, seminal receptacle condition, and parasites.

Collection of Materials. Living and dead oysters, *Crassostrea virginica*, and mussels, *Guekensia* (= *Modiolus) demissa* and *I. obsoleta*, were collected on the east side of Piver's Island and in the Rachael Carson Estuarine Research Reserve. The old valves were collected from shell piles and were intact, not fouled, and had been bleached by the sun. Egg capsules of *Busycoon carica* were collected from the Carrot Island embayment in late November of the previous fall after the opercula had opened and juvenile whelks had crawled out. The whelk egg capsules used in experiments were empty, visibly clean, and intact save the loss of the capsule operculum. Capsules were stored dry at 20–25°C until use. Mud snail egg capsules used in field experiment 2 were intact and contained living embryos in a variety of stages of development.

Assays of Snail Responses to Odors. Ring assays (Duval et al., 1994; Rahman et al., 2000) tested snail responses to odors. A 1-cm-wide $\times$ 4-cm-diam. ring of odor solution was made in a dry finger bowl using a cotton swab. A snail was placed in the center of the ring and its response upon contact was recorded. Snails either moved through the ring without hesitation (no response), turned and followed

(follow), or stopped and turned around (alarm). Thirty snails were tested with each odor solution. Frequencies of responses of following and not following were compared by Z statistic (Walpole, 1974). On each day of testing, 300–400 snails were collected at low tide from near egg capsule deposition areas on NOAA beach and stored damp in a plastic shoebox with lid.

Odors were prepared by soaking approximately equal masses of living substrates in aged filtered seawater (Rittschof et al., 1983). Aged seawater was ambient sea water that had first been filtered to remove particles >1 μm and aged for a week with aeration at room temperature and then passed through a septic 100-kDa filter and aged for at least a month in the dark before use (Rittschof et al., 1984). Living odor sources were a 7.5-cm-high oyster and mussel and 20 living mud snails. Dead odor sources were a dead oyster and mussel valve. Each source was soaked in 1 l of aged, 100-kDa filtered seawater for 1 hr. Test odors were used within 1 hr of generation.

Field Experiments. The area for the two field experiments was NOAA Beach, an east facing sand flat with a population of mud snails. A rectangular test area 8×18 m was established. The length of the rectangle was parallel to the shore. The area was raked to remove all hard substrates. A 50-point grid was established with numbered surveyor flags positioned at the center of 2 square meter areas. The first experiment began in February 2000 before any egg capsule deposition was observed. Forty of the positions were occupied by 10 replicates each of 7.5-cm-high (umbo to valve edge) living oysters and living mussels. The 7.5 cm-high-old dead oyster and dead mussel valves were assigned to points on the grid by using a random numbers table. Substrates were attached to a 7.5×12.5-cm piece of plastic screen with a cable tie. Mud snails were moved so that there were none within 30 cm of each flag, and each screen was fixed in place with the flag. Screens and cable ties were fixed at the final 10 points. The experiment was inspected at low tide until there was clear evidence of egg capsule deposition on several substrates. At that point (approximately 3 weeks), all substrates were collected and egg capsules counted.

The second field experiment, similar in design to the first, was initiated a week after the first was terminated. The apparatus controls were replaced with busycon egg capsules, comparable in surface area to an oyster valve. There were eight replicates of five substrates (living oysters and mussels, shells of oysters and mussels, and whelk egg capsules). The other 10 positions were used for a qualitative assessment of the impact of mud snail egg capsules on capsule deposition. Approximately 100 mud snail capsules deposited during the first experiment screening were affixed on two replicates of each of the five substrates. Capsules were covered with fiberglass window screening to discriminate added capsules from newly deposited capsules.

Experiment 2 was initiated by moving all mud snails within 30 cm of each flag, and then each screen with each treatment was fixed in place with the flag

for that grid point. Experiment 2 was conducted for 6 d. Every day at low tide, an observer counted every mud snail within 30 cm of each flag that marked the substrates without added mud snail egg capsules. After day 6, substrates were collected, and the numbers of capsules on each substrate counted.

RESULTS

Egg Capsule Deposition. Egg capsule deposition was determined for the first 120 shell substrates encountered in mud snail areas (Table 1). The three most common shell substrates, jackknife clams, *Tagelelus* (*pelebius* and *divisus*), oysters, *C. virginica*, and hard clams, *M. mercenaria*, accounted for over 95% of the total substrates and 100% of egg capsule deposition. Egg capsule deposition was disproportionately frequent on oysters, and uncommon on jackknife clams (Null hypothesis (1) capsule deposition was proportional to the frequency of shells $G = 335$, $P < 0.001$, 2 df). Oyster shells, 20% of the total shells, accounted for over 80% of capsule deposition sites. Deposition on jackknife clams was 24-fold lower than the relative abundance of shells.

In the second census, we walked until we encountered 100 live and 100 dead oysters, noting whether or not they were sites of egg deposition. Twenty-six of the dead oysters and 83 of the living oysters were sites of deposition. Snails deposited capsules more frequently on live oysters than on dead oysters (H_o deposition on live = deposition on dead, $G = 141$, $P < 0.001$; 1 df).

In the final two censuses, January 31 and February 4, we walked areas occupied by snails and scored capsule deposition on dead oysters within 30 cm of living oysters and on dead oysters more than 30 cm from the nearest living oyster. In the first, 96 of 100 dead oysters within 30 cm of living oysters were sites of deposition. In contrast, 73 of 97 dead oysters that were more

TABLE 1. OCCURRENCE OF HARD SUBSTRATA AND THEIR USE AS SITES OF EGG CAPSULE DEPOSITION.[a]

Genus	Total		Capsules		
	N	%	*N* With	*N* Without	% With
Tagelus	73	60.8	1	72	1.4
Crassostrea (living & dead)	23	19.2	19	4	82.6
Mercenaria	19	15.8	3	16	15.8
Dinocardium	3	2.5	0	3	0
Argopectin	1	0.8	0	1	0
Anomia	1	0.8	0	1	0
Total	120	99.9	23	97	99.8

[a] F requencies of hard substrates encountered within 1 m of the observer while walking parallel to shore in an area containing spawning mud snails.

TABLE 2. SEX, INSEMINATION STATUS, AND PARASITISM OF MUD SNAILS[a]

	Males	Females		Totals
		Full SR	Empty SR	
On mud	4	32	63	99
Parasitized	0	1	28	29
Near oysters	63	36	0	99
Parasitized	0	0	0	0
Singles	9	44	37	90
Parasitized	0	6	13	19
Total	76	112	100	298
Parasitized	0	7	41	48

[a] Numbers of males, females with and without full seminal receptacles, and parasitized animals from aggregations (on mud, and near oysters) and sandy areas. SR = seminal receptacle.

than 30 cm away were sites of deposition. (H_o Deposition <30 cm = Deposition >30 cm, $G = 52$, $P < 0.001$, 1 df). In the second census, deposition was on 68 of 100 shells with capsules 30 cm, and 51 of $100 > 30$ cm away (H_o deposition <30 cm = deposition > 30 cm, $G = 12.4$, $P < 0.001$, 1 df). In total, 164 of 200 dead oysters <30 cm from living oysters and 124 of 197 dead oysters >30 cm from living oysters were sites of egg deposition. Dead oyster shells within 30 cm of living oysters were more likely to be sites of egg capsule deposition than dead shells further than 30 cm from living oysters.

Sex, Seminal Receptacle Condition, and Parasitism in Snail Microhabitats. We collected snails from three microhabitats. We determined sex ratios, whether females had full or empty seminal receptacles, and the percentages of parasitized snails (Table 2). Snail aggregations on mud were 21:1 female to male. Approximately one third of the females had full seminal receptacles, and 45% were parasitized, including one snail with a full seminal receptacle. In contrast, aggregations ratios around oysters were approximately 1:2 females to males. All females had full seminal receptacles, and there were no parasitized snails. The single snails on sand were at a ratio of 9:1 females to males. A little over half (54%) of these females had full seminal receptacles. Of the 26 single animals that were parasitized, 6 had full seminal receptacles. In the total sample, none of 76 males, 7 of 112 females with full seminal receptacles, and 41 of 100 females without full seminal receptacles were parasitized. Contingency analysis showed large differences between the sex distributions and seminal receptacle conditions between distributions. ($G = 170.7$, $P < 0.001$, 4 df). The composition of snails around oysters was different from the composition of snails on mud and sand. More parasitized snails were found in mud aggregations than among single animals on sand (29/64 vs. 19/81; $G = 7.5$, $P < 0.01$, 1 df). Copulating snails were found only in the

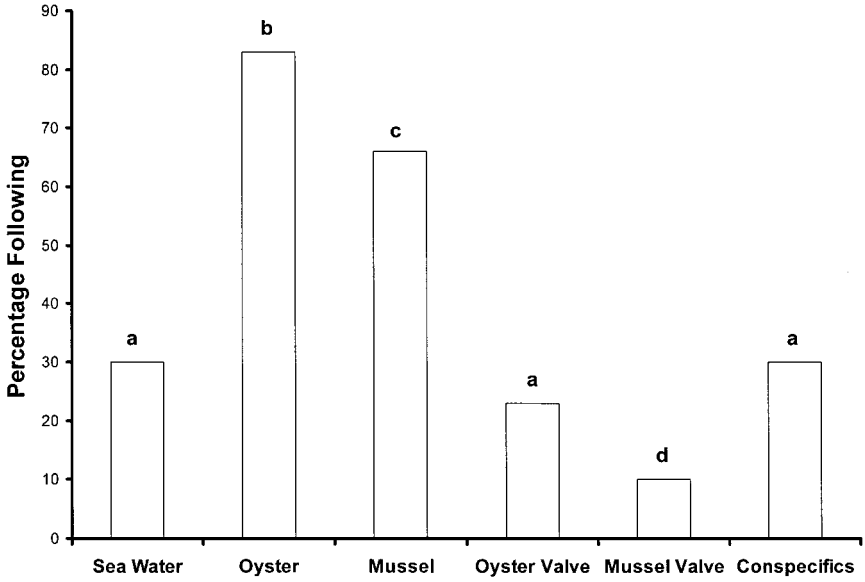


FIG. 1. Following responses of mud snails to odor water. Letters above the bars indicate similar frequencies ($P > 0.05$ same letter) and different frequencies ($P \leq 0.05$ different letters) as determined by proportions tests.

aggregations around oysters. The 12 pairs of snails were each comprised of a male and a female, and none was parasitized.

Snail Responses to Odors. Sea water incubated with living oysters, living mussels, living mud snails, and old oyster and mussel valves and plain seawater were tested for responses in ring assays (Figure 1). Three test conditions (1) seawater; (2) snail odor; and (3) oyster valve odor, resulted in the similar following responses: (9/30, 9/30, and 7/30, respectively (all Z statistics were less than 0.86; all $P > 0.1$). Dramatic increases in following responses occurred in tests with living oyster odor (25/30 for oyster odor vs. 9/30 for seawater, $Z > 10$, $P < 0.001$) and living mussel odor (20/30 for mussel odor vs. 9/30 for sea water $Z > 10$, $P < 0.01$). Mussel valve odor caused a decrease in following (3/30 for mussel valve odor vs. 9/30 for sea water, $Z = 2.6$, $P < 0.01$).

Field Experiments. In field experiment 1, the array tested contained 10 replicates each of a screen control, living oysters and mussels old oyster, and old mussel valves. There were clear differences in egg capsule deposition in the average number of capsules per substrate (Figure 2). Many more capsules were deposited on living oysters (1000s for each substrate) than were deposited on living mussels (10s per each substrate). A few capsules were deposited on several old oyster valves.

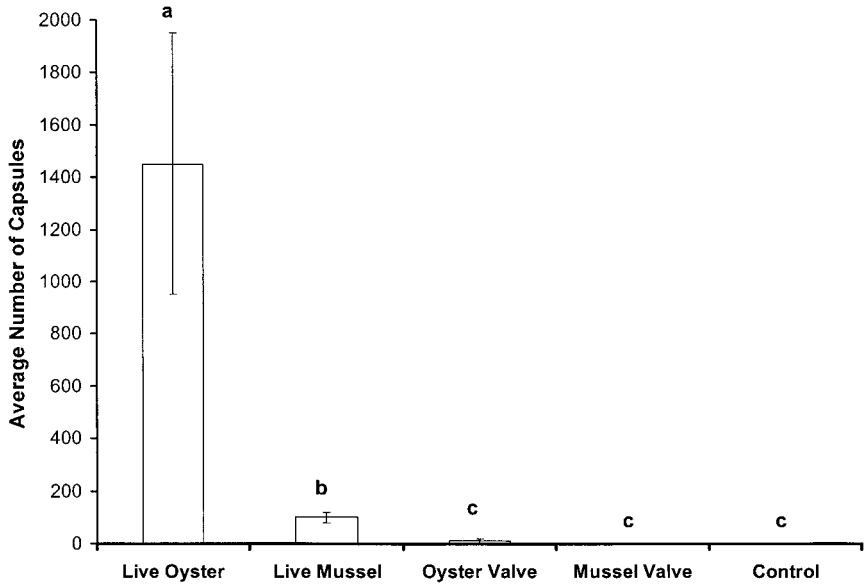


FIG. 2. Egg capsule deposition in field experiment 1. Letters above bars indicate similar ($P > 0.05$ same letter) and different ($P \leq 0.05$ different letter) as determined by ANOVA.

Deposition on oyster valves was not different statistically from mussel valves or screen controls.

In experiment 2, we counted the number of snails within 30 cm of the test substrates at low tide each day. By day 1, snail aggregations within 30 cm of whelk egg capsules, live oysters, and live mussels were developing, and they increased in number daily from an average level of 5–8 on day 1 to as many as 23 on day 6 (Figure 3). Numbers of snails around dead shells remained constant and low, averaging 2 on day 1 and less than 2 on day 6. Whelk egg capsules were most attractive, followed next by live oysters and live mussels.

Egg capsule deposition was related to aggregations. Deposition was dramatically higher on whelk egg capsules than on the other test substrates (Figure 4). Deposition of capsules on living oysters, living mussels, and on dead valves was in a proportion to similar that of experiment 1. No eggs were deposited on dead oyster or mussel valves.

The two test surfaces of each type with attached mud snail egg capsules were assessed for qualitative effects on subsequent mud snail capsule deposition (Figure 4). Mud snails deposited additional egg capsules on all substrates with attached mud snail egg capsules. Capsule deposition was at least 1.5-fold higher on all test substrates with addition of mud snail egg capsules.

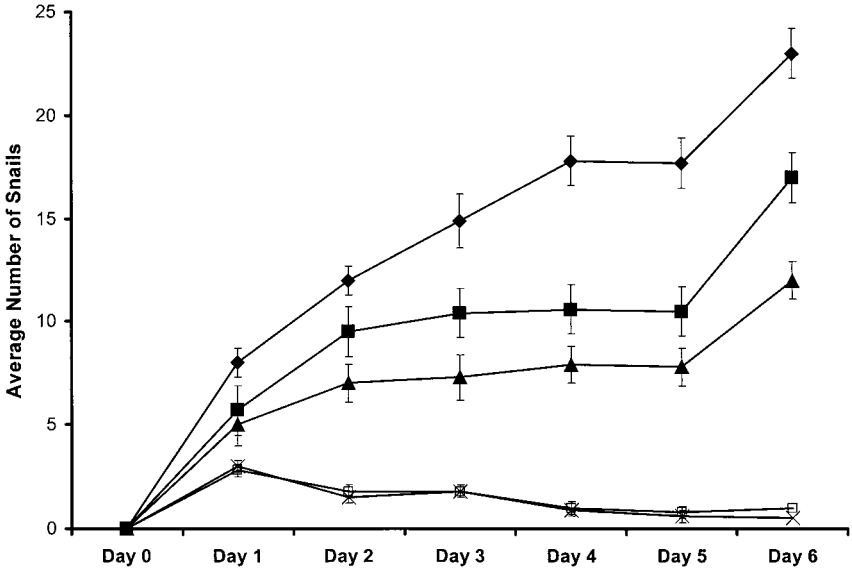


FIG. 3. Development of snail aggregations around substrates in field experiment 2. Error bars are SEM; diamonds = whelk capsules; solid squares = live oysters, solid triangles = live mussels.; open squares = oyster valves; X = mussel valves.

DISCUSSION

Field observations indicate that mud snails deposit egg capsules on the exposed valves of living bivalves. Aggregations of snails were found on mud and around living oysters. Snails were individually distributed on sandy substrates. Breeding, parasite-free snails were found in aggregations around oysters. Aggregations around oysters were 2:1 male to female snails and were a site of breeding activity. Breeding activity was not observed in mud aggregations that were 20:1 female–male snails, and about 30% of the females were parasitized. Snails from sandy substrates were similar to aggregations on mud with respect to sex composition, breeding, and parasitism. In laboratory assays, snails followed the odors of living bivalves. From 23 to 30% of the snails followed rings of sea-water, mud snail, and old oyster valve odors. Only 10% of snails followed the odor of an old mussel valve. In field experiments, mud snails were attracted to and deposited egg capsules on living bivalves, on substrates in the immediate vicinity of living oysters, and on or adjacent to mud snail and whelk egg capsules. Aggregations of snails were in response to living bivalves, mud snail egg capsules, and whelk egg capsules. We conclude that both aggregation and egg capsule deposition are chemically mediated, that both sexes are attracted, and that parasitized snails, which are

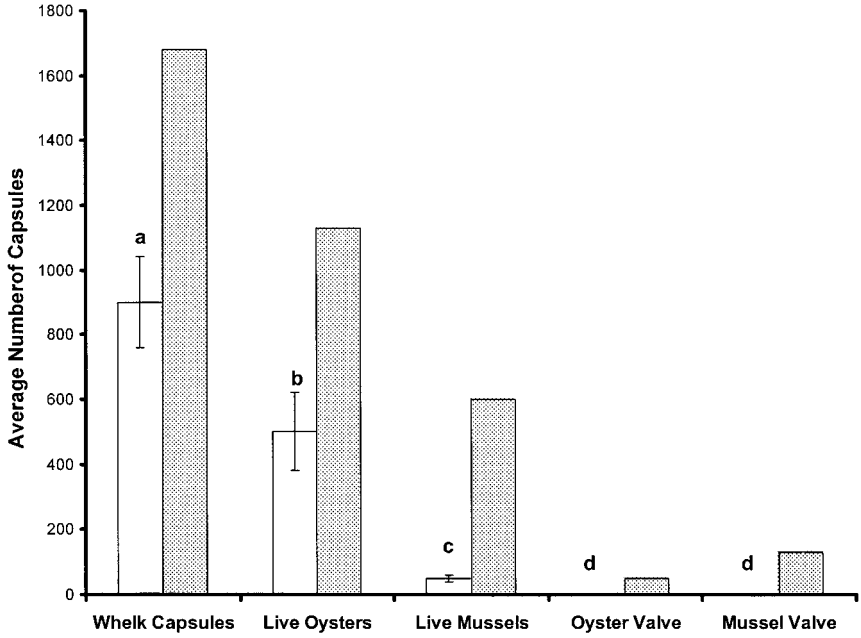


FIG. 4. Average egg capsule deposition observed in field experiment 2. Open bars are substrates ±SEM. Stippled bars are substrates with mud snail egg capsules added. Only means are presented for stippled bars because there were only two replicates of each treatment. Letters above bars indicate similar ($P > 0.05$ same letter) and different ($P \leq 0.05$ different letter) as determined by ANOVA.

functionally castrated, are unlikely to respond to these cues. The results of our studies have many similarities to observations of spawning and mating by sea hares.

Perhaps the most striking similarity between sea hare and mud snail reproduction is the coordinated stimulation of aggregation, deposition, and breeding by chemicals from eggs. In both instances, the result is large aggregations of snails, some of which are depositing eggs and others that are copulating. In both cases, the cues originate from both conspecific and heterospecific egg deposits. Cues from mud snail egg capsules might be related to egg laying hormone (ELH), as it is for sea hare egg strings (Painter et al., 1991; Painter 1992). However, if this is the case, then it is likely that the protein relative of ELH might actually be an integral part of the proteinaceous capsules housing the eggs and embryos of conspecifics and heterospecifics. This conclusion is based upon the observation that empty heterospecific capsules devoid of their embryos and nutritive contents are attractive.

For sea hares (Kupferman and Carew, 1974; Audersik, 1979; Susswein et al., 1983, 1984; Achituv and Susswein, 1985) and for mud snails, aggregations occur in

specific microhabitats. Strings and capsules are deposited in locations that ensure embryos will not be buried or washed prematurely from soft-bottomed environments. However, sea hare spawning aggregations are in sea grass, while mud snail aggregations are around oysters. This suggests that the environmental chemical cues that initiate the aggregations are different.

One option for the organization of mud snail aggregations around living bivalves is the bivalves, which are also reproductive at this time, are secreting ELH or molecules with similar activity that attract snails. Oysters and hard clams have fall and spring spawning intervals that are controlled by temperature. Thus, these animals could be attractive because they are releasing ELH-like molecules. However, if ELH-like molecules are involved, one would expect that egg-laying snails should also be producing attractive odors, as observed for sea hares (rev. Painter 1992; Painter et al., 1998,1999). In the assays conducted in 2000, we did not observe that mud snails were attractive. However, as we did not determine the sex or reproductive condition of the odor-producing or test snails, we will revisit this issue during the 2002 egg capsule deposition season. Alternatively, mud snails could be responding to odors released by bivalves that are attractive to predatory snails such as oyster drills. These odors are produced by both immature and adult bivalves (Carriker, 1955; Rittschof and Gruber, 1988; Rittschof, 1993).

Although we are convinced that egg capsule deposition by mud snails is chemically mediated, we have generated questions about the system that require more study. These questions include: revisiting the issues of aggregation and parasitism in males, studying the attractiveness of reproductive snails, determining whether not immature bivalves are attractive to mud snails, and studying the nature of responses by snails to odors in laboratory assays.

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A COMPLEX, CROSS-TAXON, CHEMICAL RELEASER OF ANTIPREDATOR BEHAVIOR IN AMPHIBIANS

DALE M. MADISON,[1,*] AARON M. SULLIVAN,[1] JOHN C. MAERZ,[2] JAMES H. MCDARBY,[1] and JASON R. ROHR[3]

[1]*Department of Biological Sciences*
State University of New York at Binghamton
Binghamton, New York 13902, USA

[2]*Department of Natural Resources*
Cornell University
Ithaca, New York 14853, USA

[3]*Department of Biological Sciences*
University of Kentucky
Lexington, Kentucky 40506, USA

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Abstract—Prey species show diverse antipredator responses to chemical cues signaling predation threat. Among terrestrial vertebrates, the red-backed salamander, *Plethodon cinereus,* is an important species in the study of these chemical defenses. During the day and early evening, this species avoids rinses from garter snakes, *Thamnophis sirtalis*, independent of snake diet, but late at night, avoids only those rinses from garter snakes that have recently eaten *P. cinereus*. We tested whether the selective, late-night response requires the ingestion or injury of salamanders. In three experiments, we tested *P. cinereus* for their responses to separate or combined rinses from salamanders (undisturbed, distressed, and injured *P. cinereus*) and snakes (unfed, earthworm fed, and salamander-fed *T. sirtalis*). When paired against a water control, only rinses from salamander-fed snakes were avoided. When salamander treatments (undisturbed or distressed) were combined with the snake treatments (unfed or earthworm-fed) and tested against a water control, the combinations elicited avoidance. When selected treatments were paired against the standard rinse from salamander-fed snakes, only the combined rinses from salamanders and snakes nullified the avoidance response to the standard rinse. These data reveal a prey defense mechanism involving chemical elements from both the predator and prey that does not require injury or ingestion of the prey in the formation of the cue.

Key Words—Chemical cues, predation, scent, composition, alarm, synergism, defense, diet, salamander, *Thamnophis sirtalis*, *Plethodon cinereus*.

* To whom correspondence should be addressed. E-mail: dmadison@binghamton.edu

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INTRODUCTION

Essentially all animals face predation threat at some point in their lives (Lima, 1998). Many of these species can significantly reduce predation risk by responding to chemosensory cues from predators or prey, with the cues varying from simple nitrogenous wastes to more specialized, damage-release chemicals stored in prey tissues (reviewed by Weldon, 1990; Kats and Dill, 1998). In some cases, combined chemical information from both the predator and prey appear to evoke a selective response in the prey species, suggesting either a complex chemical cue or multiple cues evoking a similar response. For example, prey individuals may vary their response depending on whether conspecifics occur in the predator's diet, or they may avoid both predator cues and damage-release cues (Chivers and Smith, 1998; Madison et al., 1999a,b; Murray and Jenkins, 1999; Grostal and Dicke, 2000; Chivers and Mirza, 2001). Chemosensory responses can also be context dependent, e.g., the responses vary at different times in the diel cycle (e.g., Madison et al., 1999b), with the size of the predator relative to that of the prey (Puttlitz et al., 1999), with refuge proximity (Sih and Kats, 1991), or seasonally between breeding and nonbreeding periods (Rohr et al., 2002). Despite the large number of species known to respond to chemical traces associated with predation, and despite the well-documented instances of damage-release cues particularly in aquatic species (reviewed by Chivers and Smith, 1998; Chivers and Mirza, 2001), surprisingly little is known about cue components and complexity, especially in terrestrial vertebrates (Kats and Dill, 1998).

The antipredator responses of plethodontid salamanders, especially *Plethodon* sp., have received considerable attention recently (e.g., Cupp, 1994; Lutterschmidt et al., 1994; Chivers et al., 1997; Madison 1999a,b; Murray and Jenkins, 1999; Graves and Quinn, 2000; Hucko and Cupp, 2001; Maerz et al., 2001; Sullivan et al., 2002), and this group offers a good opportunity for elucidating the source and complexity of the chemical cues signaling predation threat among terrestrial vertebrates. Their activity at night when higher relative humidity favors chemoreception (Madison, 1977), their elaborate chemosensory receptors and accessory structures (Madison, 1977), their behavioral sensitivity to a wide range of chemicals related to food, predation, social interactions, and interspecific recognition (Jaeger, 1986; Chivers et al., 1997), their diel shift from a chemically mediated daytime and early evening antipredator response to a late-night response (Madison et al., 1999b), their compromised foraging at night in the presence of predator chemical cues (Maerz et al., 2001), their avoidance of predator chemical cues in both laboratory and field studies (Sullivan et al., 2002); and their relative abundance and accessibility in many woodland habitats (Burton and Likens, 1975), collectively make them suitable for comprehensive studies of chemosensory defenses.

In the context of predation, the genus *Plethodon* appears to respond to four types of chemical cues that are often combined or confused in experimental studies:

(1) cues exclusively from predators (Madison et al., 1999a,b; McDarby et al., 1999; Murray and Jenkins, 1999), (2) "alarm" signals from wounded conspecifics and/or heterospecifics (Chivers et al., 1997; Hucko and Cupp, 2001), (3) "distress" chemicals from stressed, but not injured, conspecifics (Graves and Quinn, 2000; Mathis and Lancaster, 1998), and (4) cues from predators that have recently fed on conspecific prey (Madison et al., 1999a,b; Murray and Jenkins, 1999; Maerz et al., 2001; Sullivan et al., 2002). Cues in the fourth or "diet-specific" category are of interest because of the chemical involvement of organisms belonging to different taxa. These cues are also potentially problematic because they can easily be confused with alarm, distress, or other possible "labeling" substances transferred from prey to the predator that involve a chemical produced from just one source, in this case the prey.

In an effort to clarify the nature of the cue in the diet-specific category, single-source cues, such as alarm substances, must be shown not to be present or sufficient by themselves to elicit the response thought to result from complex, cross-taxon cues. If multi-species involvement in chemical cue production is demonstrated, we theorize three ways that true cross-taxon cues with both predator and prey involvement could be produced. The first requires the gastrointestinal digestion or alteration of a prey substance into a new product that is gradually secreted or excreted from the predator, eliciting avoidance when the chemical is encountered in the environment. A second possibility is that external integumentary or other bodily secretions from both the predator and prey chemically react during an encounter, forming a warning product that is rubbed off along the predator's path. The third possibility is a compositional, nonreactive chemical mixture of chemical products from both prey and predator, where the products only elicit avoidance when they occur together in the same chemical trace.

One of the first steps in distinguishing among the "diet-specific," cross-taxon pathways of cue production is to determine whether the ingestion of prey by the predator is required to generate the chemical cue that elicits a response among conspecific prey, i.e., is diet-specific antipredator behavior really "diet" specific? We approached this problem by collecting rinses from garter snakes and red-backed salamanders and then testing whether salamanders would avoid these rinses only when they were combined. We speculated that late at night when only rinse from salamander-fed snakes is avoided, *P. cinereus* would avoid a rinse of unfed or earthworm-fed snakes combined with undisturbed or noninjured salamanders, but would not avoid the snake or salamander rinses tested separately.

METHODS AND MATERIALS

A total of 416 adult salamanders [*P. cinereus*; snout/vent length (SVL) $>$ 40 mm] and 8 adult garter snakes (*T. sirtalis*; SVL $>$ 36 cm) were collected from

the Binghamton University Nature Preserve (Broome Country, New York, USA). Salamanders and snakes were housed in separate rooms at 15°C (salamanders) and 25°C (snakes) on a natural photoperiod (15L:9D). Salamanders were housed individually in 15-cm diam Petri dishes lined with moistened paper towels. Each salamander was tested once within 10 d of capture and then released at the capture site. Snakes were housed individually in 38-l glass aquaria, provided with a heating block, given crumpled paper towels for cover, and maintained on a diet of *P. cinereus* or earthworms (*Lumbricus* sp.) before and during the experiment. They were released following the experiments.

Salamanders were subjected to distilled water rinses of different snake and salamander treatments or to distilled water only, in two-choice, substrate tests. The methods for preparing treatments and testing salamander avoidance are similar to those of Madison et al. (1999b), with the modifications described below.

Experiment I. Trials with Distressed Salamanders and Earthworm-Fed Snakes. Four treatment rinses were prepared: salamander-fed snake, earthworm-fed snake, distressed salamander, and a combination of the latter two. The salamander-fed snake treatment is a standard treatment used in all our experiments to provide a measure of whether recently captured salamanders are behaving similarly to previously tested salamanders. The rinses from the earthworm-fed snake and the distressed salamander were tested singly and then together against distilled water to determine whether the combination rinse, but not the independent rinses, would elicit avoidance.

The salamander-fed snake rinse consisted of a 200-ml rinse of an adult garter snakes (snake 1) that had been maintained on a diet of 4 *P. cinereus*/w for 2 w prior to sample collection, with 4 of these salamanders (2 male, 2 female) being fed to the source snake just prior to sample collection. Although individual snakes may differ at least in some of their chemical secretions (Oldak, 1976), previous research has shown that the response of individual *P. cinereus* to snake chemical cues does not noticeably vary with the snake used to collect the test rinses (Madison et al., 1999b). In addition, rinses from 18 *T. sirtalis* that have been fed *P. cinereus* over the past 7 yr have never failed to elicit avoidance in *P. cinereus* (Madison, Sullivan, Maerz, McDarby, personal observation). Collection of the snake rinses entailed transferring the snakes to individual 4-l beakers, covering them with 8 layers of cheesecloth secured by rubber bands, placing a heating pad (26°C) under half the beaker base, waiting 72 hr, carefully removing the snake, and then collecting a 200-ml distilled water rinse of all smears, secretions, and excretions deposited in the beaker.

The earthworm-fed snake treatment rinse was identical to the above, except that earthworms equivalent in mass to the salamanders were fed to an adult garter snake (snake 2). The distressed salamander treatment rinse consisted of a 200-ml sample of distilled water in which 4 adult *P. cinereus* (2 male, 2 female) had been held by blunt forceps and agitated for 2 min each until defensive

secretions were released from their tails and body (but where no wounding had occurred).

We filtered all rinses through a 0.45-μm filter to remove solid materials, and divided the filtrate into four 50-ml aliquots. The fourth treatment (combination treatment) was prepared by combining 50-ml aliquots of the earthworm-fed snake and the distressed salamander rinses. The resulting combination rinse, therefore, had half the concentration of each of the two separate components. All four treatments were rapidly frozen in liquid nitrogen, stored in a freezer, and thawed at room temperature just prior to use. Sullivan et al. (2002) showed that salamanders still avoid salamander-fed snake rinse that has been frozen using this method.

For testing (Madison et al., 1999a,b; Sullivan et al. 2002), we placed two filter paper semicircles with a 3-mm gap between them into 15-cm Petri dishes, inoculated the semicircles with 1.5 ml of treatment rinse or distilled water, and randomly placed these test dishes (position and orientation) in an 8×7 pattern on the floor of the test room. A total of 168 salamanders were tested once beginning just after midnight over three consecutive nights. On each night, 14 replicate dishes were prepared for each treatment comparison, giving 14 replicates for each of the four comparisons per night. Thus, 56 salamander dishes were distributed at random and tested in a 8×7 pattern each night. Salamanders were moved to the test room 1 hr prior to the experiments, which began between 24:00 and 01:00 hr. We transferred each salamander under dim red light from the home dish to the test dish with a cotton swab, and placed a 15-mm collar of brown paper around the covered dish to isolate each salamander visually during the transfer process. After all salamanders were distributed ($\sim$10 min), the lights were turned off and the trial was recorded with an infrared, 0-lux, video camera (Sony TRV66) suspended 3 m above the dishes. We scored which semicircle the salamander occupied at 3-min intervals for 1 hr beginning a time 0, which gave 21 positions per salamander. A score of 10 or less for a treatment was recorded as avoidance. Where salamanders straddled the 3-mm gap, two criteria were used to judge side position. If the salamander was moving, the side into which the salamander was moving was scored. If the salamander was not moving, the side occupied by more than half the body, measured from the midpoint of the trunk, was scored. All scoring was performed by an observer blind to treatment type and right/left side in the test dishes. We used a chi-square goodness-of-fit test to determine whether salamander responses to treatments departed significantly from random expectation (Siegel and Castellan, 1988).

Experiment II. Trials with Distressed Salamanders and Unfed Snakes. In the previous experiment, it is possible that any avoidance elicited by the combined rinse of earthworm-fed snake and distressed salamander might have nothing to do with an interaction of snake and salamander chemicals, and instead be a response to an interaction between chemicals from earthworms and salamanders. Experiment II repeats experiment Experiment I, except rinses from unfed snakes replace those

from earthworm-fed snakes, and two snakes are used for each snake rinse. If only the standard and combined rinses elicit avoidance, the results would more clearly indicate that neither ingestion nor injury of the salamander is required to elicit avoidance, and that chemicals from both noninjured salamanders and snakes are required.

Seven treatments were prepared and tested. Treatments A and B consisted of a 200-ml rinse from two different adult garter snakes (snakes 3 and 4) that had not been fed for 3 wk prior to sample collection, a period sufficient to negate a diet effect (Madison et al., 1999b). The use of different snake sources for treatments A and B, and two snake sources for other treatments mentioned below, was an effort to provide an additional test of possible snake source effects, as referred to in experiment 1. Treatment C consisted of rinse from distressed *P. cinereus*, collected in the same manner as in experiment I. Treatments D and E consisted of salamander-fed snake rinse from two different adult garter snakes (snakes 5 and 6) that had been treated identically to the comparable treatment in experiment 1. Treatments F and G were combination treatments made up of equal portions of treatments A and C and of B and C, respectively. The combined treatments were prepared as in experiment 1.

Testing was performed as previously stated. A total of 168 salamanders were tested once just after midnight over three consecutive days. On each night, 7 replicate dishes were prepared for each treatment, except for treatment C (distressed salamander), which was represented by 14 replicated dishes (giving 14 replicates for each of the four main treatments per night). Thus, 56 salamander dishes were distributed at random and tested each night, as in experiment 1.

We used a χ^2 goodness-of-fit test to determine whether salamander responses to treatments departed significantly from random expectation, and then a 2×2 χ^2 contingency test to look for differences between snake sources and between treatments (Siegel and Castellan, 1988). Interactions between *T. sirtalis* and *P. cinereus* can only be inferred using chi-square tests, and since this experiment with unfed snakes was the clearest demonstration of salamander and snake component effects, we used binomial regression with a log–log canonical link in Statistica's (Statsoft, Inc., 1998 version 5.5A) Generalized Linear model (GLZM) to quantitatively test for the predicted snake by salamander interaction. We chose to test for significance of this interaction using the Wald statistic, rather than likelihood ratios, due to its ease and efficiency of computation (Dobson, 1990).

Experiment III. Selected Individual and Combination Treatments Tested Against Rinses from Salamander-Fed Snakes. The previous experiments left unresolved several questions: (1) whether always giving salamanders a choice of moving to nonsoiled substrates would affect their response to treatment substrates, (2) whether salamanders would discriminate between the substrates of two snakes on different diets, and (3) whether the distressed salamanders may have elicited a subtle but nonsignificant avoidance effect that would still act as a prey label in

the combined tests. In a single attempt to address these questions, we paired our standard salamander-fed snake rinse against four treatment rinses: earthworm-fed snake, injured salamander, earthworm-fed snake + undisturbed salamander, and earthworm-fed snake + injured salamander.

Treatment rinses were collected as before, except that two new treatments were prepared: undisturbed salamander and injured salamander. Undisturbed-salamander rinse was collected by housing two salamanders (one male and one female) in separate Petri dishes on moistened filter paper for the 72 hr during which time snake chemical cues were accumulating for the snake rinses. After 72 hr, the filter paper was removed from both Petri dishes and placed in 200 ml of distilled water. The injured salamander rinse was collected using the two undisturbed salamanders above in a two-step procedure. First, they each were immersed in the same 200 ml of distilled water during which they were physically disturbed, identical to the collection of distressed salamander rinse previously described. Second, they were removed, decapitated, and homogenized in the same 200 ml of distilled water with the distress substances. Both rinses were passed through glass wool before use in preparing the test dishes. All tests were performed beginning at midnight immediately after treatment preparation, and all observations were performed and analyzed as in previous studies (Madison, 1999a,b). We used a chi-square goodness-of-fit test to determine whether salamander responses to the different treatments departed significantly from random expectation (Siegel and Castellan, 1988).

RESULTS

The first experiment showed that *P. cinereus* did not avoid rinses from earthworm-fed snake or the distressed salamander when these were tested separately against distilled water in late-night trials. However, salamanders significantly avoided the standard salamander-fed snake rinse ($\chi^2 = 13.71$, $df = 1$, $P < 0.001$). The combined rinse of earthworm-fed snake and distressed salamander was also avoided ($\chi^2 = 7.71$, $df = 1$, $P = 0.005$), despite the concentrations of the components in the combined rinse being half those in the separate rinses (Table 1).

In the second experiment, we first looked for differences in response to the two unfed snakes when tested alone (treatments A and B) or in combination with salamander rinse (treatments F and G). No difference occurred between treatments A and B ($\chi^2 = 0.86$, $df = 1$, $P = 0.35$; Table 2) or between F and G ($\chi^2 = 0.12$, $df = 1$, $P = 0.73$). In addition, no difference in response was given to the two snakes that were fed salamanders (treatments D and E; $\chi^2 = 0.00$, $df = 1$, $P = 1.00$). Thus, the results for each snake source within each treatment were combined for further analyses.

TABLE 1. NUMBER OF SALAMANDERS PREFERRING SUBSTRATES WITH DISTILLED WATER VS. SUBSTRATES WITH DISTILLED WATER RINSES[a]

Trial type	Salamanders preferring (N): Treatment	Water	$\chi^2 b$	P
Ts_{Pc} vs. water	9	33	13.71	<0.001
Ts_W vs. water	17	25	1.52	0.217
Pc_D vs. water	20	22	0.10	0.758
$Ts_W + Pc_D$ vs. water	12	30	7.71	0.005

[a] Rinses were of *T. sirtalis*-fed *P. cinereus* (Ts_{Pc}), *T. sirtalis*-fed earthworms (Ts_W), distressed *P. cinereus* (Pc_D), or Ts_W and Pc_D Rinses Combined ($Ts_W + Pc_D$)
[b] $df = 1$.

The unfed snake treatments (A + B) elicited no avoidance behavior in salamanders during the late-night trials ($\chi^2 = 0.00$, $df = 1$, $P = 1.00$; Table 2), nor did the distressed salamander rinse ($\chi^2 = 0.38$, $df = 1$, $P > 0.50$). However, the salamander-fed snake treatments (D + E) elicited significant avoidance ($\chi^2 = 16.10$, $P < 0.001$), as did the combination treatments (F + G; $\chi^2 = 9.52$, $df = 1$, $P < 0.01$). These results corroborate the results for those treatments that were equivalent in experiment 1 and suggest that natural body secretions or excretions from undisturbed salamanders are sufficient to elicit avoidance when combined with rinses from recently foraging (on earthworms) or nonforaging snakes.

TABLE 2. NUMBER OF SALAMANDERS PREFERRING SUBSTRATES WITH DISTILLED WATER VS. SUBSTRATES WITH DISTILLED WATER RINSES[a]

Trial type	Treatment	Salamanders preferring (N): Treatment	Water	χ^2	P
Ts vs. water	(A) Ts_1	12	9		
	(B) Ts_2	9	12		
		21	**21**	0.00	1.00
Pc_D vs. water	(C) Pc_D	**19**	**23**	0.38	>0.50
Ts_{Pc}vs. water	(D) Ts_{3Pc}	4	17		
	(E) Ts_{4Pc}	4	17		
		8	**34**	16.10	<0.001
Ts + Pc_D vs. water	(F) $Ts_1 + Pc_D$	6	15		
	(G) $Ts_2 + Pc_D$	5	16		
		11	**31**	9.52	<0.01

[a] Rinses were of unfed *T. sirtalis* (Ts), distressed *P. cinereus* (Pc_D), *T. sirtalis*-fed *P. cinereus* (Ts_{Pc}), or Ts and Pc_D combined (Ts + Pc_D). Numbers following Ts refer to the different snakes used. Chi-square results are given for within-trial comparisons ($df = 1$ for each comparison); no differences occurred between snakes within trial types.

Comparing the results of the four main treatments, there was no difference between unfed snake and distressed salamander treatments (χ^2 2 × 2 contingency test, $\chi^2 = 0.19$, $df = 1$, $P = 0.66$), but there was a difference between rinses from unfed snakes vs. salamander-fed snakes ($\chi^2 = 8.90$, $df = 1$, $P = 0.003$) and between unfed snakes vs. the combination of unfed snakes and distressed salamanders ($\chi^2 = 5.10$, $df = 1$, $P = 0.03$). There was a significant difference between distressed salamanders and the salamander-fed snake standard ($\chi^2 = 6.60$, $df = 1$, $P = 0.01$), but a marginally insignificant difference between distressed salamanders and the combined unfed snake + distressed salamander treatment ($\chi^2 = 3.30$, $df = 1$, $P = 0.068$). Finally, there was no difference between the standard rinse and the combined unfed snake + distressed salamander ($\chi^2 = 0.61$, $df = 1$, $P = 0.43$).

Since there was no independent avoidance of unfed snake rinse or distressed salamander rinse, but a significant avoidance of these treatments combined, a snake × salamander interaction was suggested. We combined the data for the standard rinse and the combination rinse of distressed salamander + unfed snake because both contained chemicals from stressed salamander and snake, and then we compared these against the independent results for snakes (treatments A and B) and distressed salamanders (treatment C). An unfed snake × distressed salamander interaction was confirmed using binomial regression ($\text{Wald}_{1,206} = 5.42$, $P = 0.02$).

Finally, in experiment 3, the results of the dual treatment tests gave results consistent with previous predictions and findings for individual treatment tests against a distilled water control. Rinses from standard salamander-fed snake treatments were avoided relative to earthworm-fed snake ($\chi^2 = 5.00$, $df = 1$, $P = 0.03$) and to injured (homogenized) salamander treatments ($\chi^2 = 7.20$, $df = 1$, $P < 0.01$), indicating that neither earthworm-fed snake rinses nor injured salamander rinses were as aversive as the standard rinse (Table 3). The lack of a difference between the combined samples and the standard salamander-fed snake rinse suggests that *P. cinereus* finds these treatments equally undesirable.

DISCUSSION

The results corroborate previous findings that rinses from *P. cinereus*-fed *T. sirtalis* consistently elicit avoidance in laboratory trials when compared to water controls (e.g., Madison et al. 1999a,b; Maerz et al., 2001; Sullivan et al., 2002), regardless of different source snakes being used, and that earthworm-fed snake rinse and unfed snake rinse fail to elicit avoidance in late-night tests when salamander-fed snake rinse continues to elicit avoidance (Madison et al., 1999a,b). The results also show that, under the conditions in our study, rinse from distressed *P. cinereus* repeatedly fails to elicit avoidance. When the combined rinses from distressed

TABLE 3. NUMBER OF SALAMANDERS PREFERRING SUBSTRATES WITH DISTILLED WATER RINSES OF *T.sirtalis*-FED *P.cinereus* (Ts_{Pc}) VS. DISTILLED WATER RINSES OF *T. sirtalis*-FED EARTHWORMS (Ts_W), HOMOGENIZED *P.cinereus* (Pc_H), Ts_W COMBINED WITH UNDISTURBED *P. cinereus* (Pc) (Ts_W + Pc), AND Ts_W COMBINED WITH Pc_H ($Ts_W + Pc_H$).

Trial type	Salamanders preferring (*N*): Treatment	Ts_{Pc}	Ties	χ^{2a}	*P*
Ts_W vs. Ts_{Pc}	15	5	0	5.00	0.03
Pc_H vs. Ts_{Pc}	16	4	0	7.20	<0.01
Ts_W + Pc vs. Ts_{Pc}	10	9	1	0.05	0.82
$Ts_W + Pc_H$ vs. Ts_{Pc}	7	13	0	1.80	0.18

[a] $df = 1$.

P. cinereus and unfed or earthworm-fed *T. sirtalis* elicited avoidance late at night, two things became clear. First, the cue eliciting avoidance behavior must require chemical involvement of both the predator and the prey. Second, neither ingestion by the predator nor distress or alarm substances from disturbed or injured prey is necessary to elicit late-night avoidance in *P. cinereus*.

There are two possible explanations for the above findings: (1) substances from the snake and salamander remained active in the separate rinses and chemically reacted to produce a novel "warning" substance when the rinses were combined, or (2) chemicals from snakes and salamanders only evoke a response when they occur as a mixture in the same scent trace, and in this case, the cue would be compositional rather than being a novel chemical product. It is difficult to determine which of these two hypotheses is more likely. In support of both is that during snake attack, *P. cinereus* usually deposits milky tail secretions directly to the snake's head, and that after ingestion or prey escape, the film remains and the snake goes through head rubbing on the substrate for a few minutes. This behavior has also been reported for *T. sirtalis* in response to the adhesive mucus of earthworms (Cunningham and Burghardt, 1999). Not only is there potential mixing of substances from salamander and snake, but the subsequent effort by the snake to free its integument (eyes, scales) of the gluey matrix would facilitate the deposit of substances in this matrix on the nearby landscape.

The above scenario for a novel chemical cue or a composite cue used by *P. cinereus* late at night does not preclude the concurrent existence of multiple chemical mechanisms involved in antipredator behavior in this species. In fact, the avoidance of any *T. sirtalis* trace during the day or early evening, but only of *P. cinereus*-fed *T. sirtalis* rinses late at night, indicates two mechanisms with temporal dependence (Madison et al., 1999a,b). In addition, other data show a third mechanism in *P. cinereus*: the late-night avoidance of damage-release "alarm" substances (Sullivan, Madison and Rohr, unpublished reults). Damage-release

cues, by themselves, are probably not responsible for the avoidance of the standard salamander-fed snake rinses late at night, since in the present study these alarm cues failed to cancel the effect of the standard rinses in eliciting avoidance. Thus, the new chemical mechanism, which does not require tissue injury, could be the basis for the avoidance of both the combination rinses and the standard rinse from salamander-fed snakes. Whatever the ultimate explanation is, at least four different chemical mechanisms are used by *P. cinereus* in the context of predator avoidance, with the fourth or "dietary" mechanism requiring chemicals from both the predator and prey and being either a compositional mixture or a novel cue formed by a non-ingestive chemical reaction between superficial rinse products from both predator and prey.

Our study is the first demonstration of antipredator behavior being released by a cue where substances from both the predator and prey are required to elicit a response. How common such complex, "cross-taxon" cues may be in nature is unknown, but our findings strongly suggest that future studies should not restrict the search for chemical cues that elicit antipredator behavior to substances released from just the prey or predator.

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(*Z*,*Z*)-4,7-TRIDECADIEN-(*S*)-2-YL ACETATE: SEX PHEROMONE OF DOUGLAS-FIR CONE GALL MIDGE, *Contarinia oregonensis*

REGINE GRIES,[1] GRIGORI KHASKIN,[1] GERHARD GRIES,[1,*] ROBB G. BENNETT,[2] G. G. SKIP KING,[1] PETRA MOREWOOD,[1] KEITH N. SLESSOR,[3] and W. DEAN MOREWOOD[1]

[1]*Centre for Environmental Biology*
Department of Biological Sciences, Simon Fraser University
Burnaby, British Columbia, V5A 1S6 Canada

[2]*Ministry of Forests*
Tree Improvement Branch
Saanichton, British Columbia, V8M 1W4 Canada

[3]*Department of Chemistry*
Simon Fraser University
Burnaby, British Columbia, V5A 1S6 Canada

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Abstract—Our objectives were to identify and field test the sex pheromone of female Douglas-fir cone gall midge, *Contarinia oregonensis* (Diptera: Cecidomyiidae). Coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses of pheromone extract revealed a single compound (**A**) that elicited responses from male antennae. Hydrogenation of pheromone extract, followed by renewed GC-EAD analysis, revealed a new EAD-active compound with chromatographic characteristics identical to those of tridecan-2-yl acetate on five fused silica columns (DB-5, DB-210, DB-23, SP-1000, and Cyclodex-B). Syntheses, chromatography, and retention index calculations of all possible tridecen-2-yl acetates suggested that the candidate pheromone **A** was a tridecadien-2-yl acetate with nonconjugated double bonds. Synthetic candidate pheromone component (*Z,Z*)-4,7-tridecadien-2-yl acetate (*Z*4*Z*7) cochromatographed with **A** on all analytical columns and elicited comparable antennal activity. In GC-EAD analyses that separated the enantiomers (*Z,Z*)-4,7-tridecadien-(*S*)-2-yl acetate (2*S*-*Z*4*Z*7) and (*Z,Z*)-4,7-tridecadien-(*R*)-2-yl acetate (2*R*-*Z*4*Z*7) with baseline resolution, only 2*S*-*Z*4*Z*7 as a component in a racemic standard or in pheromone extract elicited antennal responses. In Douglas-fir seed orchards, sticky traps baited with 2*S*-*Z*4*Z*7 captured

* To whom correspondence should be addressed. E-mail: gries@sfu.ca

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male *C. oregonensis*, whereas 2*R*-*Z*4*Z*7 was behaviorally benign. Comparable catches of males in traps baited with racemic *Z*4*Z*7 (50 μg) or virgin female *C. oregonensis* suggested that synthetic pheromone baits could be developed for monitoring *C. oregonensis* populations in commercial Douglas-fir seed orchards.

Key Words—Douglas-fir cone gall midge, *Contarinia oregonensis*, Cecidomyiidae, sex pheromone, enantiomers, (*Z*,*Z*)-4,7-tridecadien-(*S*)-2-yl acetate, (*Z*,*Z*)-4,7-tridecadien-(*R*)-2-yl acetate.

INTRODUCTION

The life history of the Douglas-fir cone gall midge, *Contarinia oregonensis* Foote (Diptera: Cecidomyiidae) is closely associated with the phenology of its host plant Douglas-fir, *Pseudotsuga menziesii*. In early spring as conelets open for pollination, female *C. oregonensis* eclose from their pupae in the duff, mate, and seek open conelets for oviposition. Females place their eggs singly or in clusters near the base of cone scales by extending their flexible ovipositor. Neonate larvae tunnel into cone scales and induce formation of, and develop within, galls near ovules. In the fall, larvae leave mature cones and overwinter in the duff as prepupae or pupae (Hedlin, 1961).

In natural forests, *C. oregonensis* is one of many insects that feed and develop on seeds. However, in coastal seed orchards of British Columbia, in Canada and Washington and Oregon, in the United States, *C. oregonensis* has become the single most important insect pest of Douglas-fir seeds. Larvae-caused gall formation results in seed fusion to the scale, preventing seed extraction at harvest or destruction of seeds at high populations. Severe infestations destroy the entire seed crop (Hedlin, 1961, 1974; Ruth, 1980).

C. oregonensis is the only coastal Douglas-fir seed orchard pest in British Columbia monitored annually. Monitoring entails tedious and time-consuming counts of egg-infested scales in samples of conelets. When a threshold number of infested scales per conelet is reached, insecticidal control of *C. oregonensis* is implemented.

Miller and Borden (1981, 1984) presented evidence for a sex attractant pheromone produced by female *C. oregonensis.* Traps baited with synthetic *C. oregonensis* pheromone would allow investigation of relationships between captures of male *C. oregonensis* and numbers of both egg-infested scales at pollination and galled scales at harvest. If reliable correlations could be established, decisions about *C. oregonensis* control could be based solely on captures of males in pheromone-baited traps. Monitoring of *C. oregonensis* would become simple and effective.

We report identification and field testing of the pheromone.

METHODS AND MATERIALS

Experimental Insects. In late August and early September 1995–1996, mature, dry Douglas-fir cones infested with larvae of *C. oregonensis* were collected into loosely woven burlap sacks. These sacks were then partially submerged in water-filled pails for 2–3 days to induce the mature larvae to leave the cones. After removing the sacks from the pails, the larvae that had exited the cones and sacks and accumulated in the bottom of pails (apparently unaffected by their temporary submergence) were obtained by pouring the water through a No. 30 standard sieve (600 μm). Sieve-restrained larvae were then transferred to 500-ml glass jars half filled with clean, damp sand and closed with fine mesh screening. To break larval developmental diapause, jars were placed in environmental chambers (18°C; 70% relative humidity.) with temperature lowered by 2°C every 5 d to 4°C. After 3.5 months of cold exposure (4°C), jars were subjected to a temperature increase from 4 to 18°C in increments of 2°C every 5 d. Emergent adults were collected several times a day and kept individually in Petri dishes. Ovipositors (with pheromone glands) of calling females and abdominal tips of males were removed and separately extracted in hexane for 5–50 min.

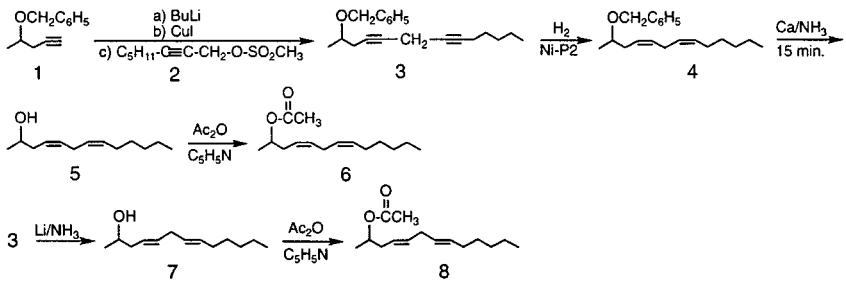
Laboratory Analyses of Pheromone Extract and General Instrumentation. Aliquots of 5–10 female and male equivalents (FE, ME) of abdominal tip extract were analyzed by gas chromatography (GC) and coupled gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975), employing an Hewlett-Packard (HP) 5890 gas chromatograph fitted with a fused silica column (30 m $\times$ 0.25 or 0.32 mm ID) coated with DB-210, DB-5, DB-23, Cyclodex-B (J&W Scientific, Folsom, California, USA) or SP-1000 (Supelco, Bellefonte, Pennsylvania, USA). For GC-EAD recordings, a severed insect head (with both antennae intact) was placed into the opening of a glass capillary electrode (1.0 $\times$ 0.58 $\times$ 100 mm) (A-M Systems, Inc., Carlsborg, Washington, USA) filled with saline solution (Staddon and Everton, 1980). One antenna with its tip removed by spring microscissors (Fine Science Tools Inc., North Vancouver, British Columbia, Canada) was placed into the recording capillary electrode. Coupled GC-mass spectrometric (MS) analyses of pheromone extract (300 FE) and of synthetic standards employed a Varian II Ion Trap GC-MS fitted with the above-referenced DB-5 column.

High-performance liquid chromatography (HPLC) employed a Waters LC 626 high-performance liquid chromatograph equipped with a Waters 486 variable wavelength UV visible detector set to 210 nm, HP Chemstation software (Rev.A.07.01), and a normal phase Nova-Pak silica column (60Å, 4 μm; 3.9 $\times$ 150 mm) with 1 ml/min of hexane–ether (97:3) flow. Nuclear magnetic resonance (NMR) spectroscopy of synthetic compounds was conducted on a

Bruker AMX-400 spectrometer at 400.13 MHz for ^{1}H NMR spectra. ^{1}H chemical shifts are reported as parts per million (ppm; δ) relative to TMS. Elemental analyses were performed using a Carlo Erba model 1106 elemental analyzer. Chemicals obtained from commercial sources were used without further purification unless otherwise indicated. All moisture- and air-sensitive reactions were conducted under argon. Column chromatography refers to flash chromatography, using silica gel 60 (230–400 mesh, E. Merck, Darmstadt) (Still et al., 1978).

Syntheses

*(Z,Z)-4,7-Tridecadien-2-ol (**5**) (Scheme 1).* To a THF solution of the benzyl ether of 4-pentyn-2-ol (**1**) (4.35 g, 25 mmol), 11 ml of 2.5 M butyllithium in hexane (10% excess) was added at -70°C. The mixture was stirred under argon and then warmed to -25°C before addition of CuI (475 mg, 0.1 equiv.). After stirring for 1 hr at -25°C, a THF solution of freshly prepared 2-octyn-1-yl methanesulfonate (**2**) (30 mmol) (from 2-octyn-1-ol, methanesulfonyl chloride, triethylamine, 0°C) was added dropwise. The reaction mixture was allowed to warm to room temperature, quenched with aq. conc. NH_4Cl, and after 2 hr of stirring was extracted with hexane–ether (1:1). The desired benzyl ether of 4,7-tridecadiyn-2-ol (**3**) (40% yield) was purified (85% based on GC) by flash chromatography and reduced to the benzyl ether of (*Z*,*Z*)-4,7-tridecadien-2-ol (**4**) by catalytic hydrogenation with P-2 Ni in ethanol. Flash chromatography on $AgNO_3$-impregnated (20%) SiO_2, with a gradient of hexane–ether (99:1 to 95:5) eluent afforded (*Z*,*Z*)-diene (**4**) (76% yield, 94 % pure). Removal of the benzyl group of **4** by exposure to excess calcium (15 equiv.) in liq. ammonia (Hwu et al., 1986) for 15 min at -70°C yielded (*Z*,*Z*)-4,7-tridecadien-2-ol (**5**) (71%) and corresponding monoenes (7%), as determined by GC-MS. Therefore, **5** was repurified by flash chromatography as above to give 1.05 g (5.40 mmol) of pure **5**. Anal. calcd. for $C_{13}H_{24}O$: C 79.13; H 12.22. Found: C 79.53; H 12.32. ^{1}H NMR: $CDCl_3$, δ = 5.31-5.61 (4H, m), 3.88



SCHEME 1. Synthesis of (*Z*,*Z*)- and (*E*,*E*)-4,7-tridecadien-2-yl acetates.

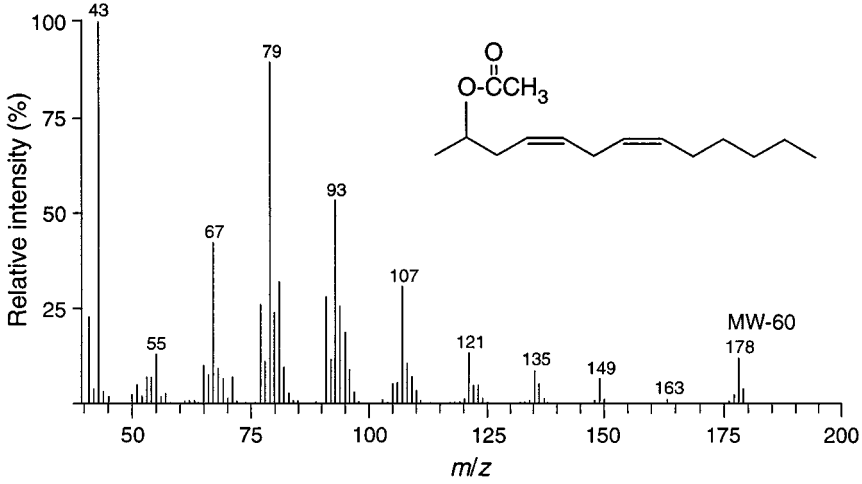


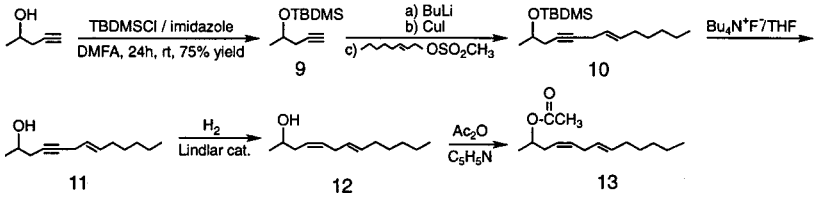
FIG. 1. Mass spectrum of synthetic (*Z*,*Z*)-4,7-tridecadien-2-yl acetate. Varian Saturn II Ion Trap GC-MS.

(1H, sextet), 2.85 (2H, t), 2.28 (2H, m), 2.08 (2H, m), 1.34 (6H, m), 1.25 (3H, d), 0.92 (3H, t).

*(Z,Z)-4,7-Tridecadien-2-yl Acetate (**6**) (Scheme 1).* Overnight reaction of **5** with acetic anhydride in pyridine afforded acetate **6** (1.20 g, 5.0 mmol) in 93% yield (20% overall yield based on **1**). Anal. calcd. for $C_{15}H_{26}O_2$: C 75.58; H 11.00. Found: C 75.80; H 10.98. ^{1}H NMR: $CDCl_3$, $\delta = 5.28$–5.54 (4H, m.), 4.92 (1H, sextet), 2.78 (2H, t), 2.31 (2H, m), 2.04 (2H, m), 2.01 (3H, s), 1.27 (6H, m), 1.22 (3H, d), 0.88 (3H, t); mass spectrum as in Figure 1.

*(E,E)-4,7-Tridecadien-2-yl Acetate (**8**) (Scheme 1).* Overnight reaction of **3** (1.13 g, 4.00 mmol) with lithium (20-fold excess) in refluxing liq. ammonia produced as the major product geometrically pure (*E*,*E*)-4,7-tridecadien-2-ol (**7**) (60% yield). It was purified by flash chromatography (see above) and acetylated as above to give (*E*,*E*)-4,7-tridecadien-2-yl acetate (**8**) (0.520 g, 2.18 mmol).

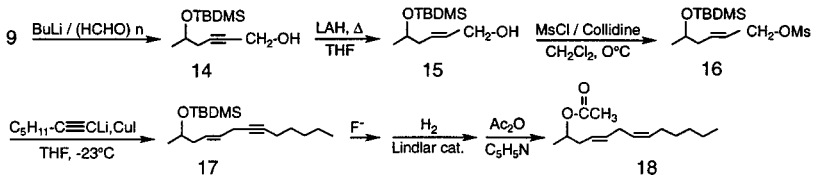
*(Z,E)-4,7-Tridecadien-2-yl Acetate (**13**) (Scheme 2).* 2-*tert*-Butyl-dimethylsilyloxy-3-pentyne (**9**) (5.14 g, 15.6 mmol) was coupled via cuprate with freshly prepared (*E*)-2-octen-1-yl methanesulfonate (15.2 mmol) (from 2-octen-1-ol, methanesulfonyl chloride, collidine at 0°C, 50% yield) in THF. The resulting crude *tert*-butyldimethylsilyl ether of (*E*)-7-tridecen-4-yn-2-ol (**10**) was de-protected with Bu_4NF in THF affording alcohol **11** (80% pure), which was hydrogenated with Lindlar catalyst in hexanes to (*Z*,*E*)-4,7-tridecadien-2-ol (**12**, 1.46 g, 7.43 mmol). Acetylation of purified **12** produced geometrically pure (*Z*,*E*)-4,7-tridecadien-2-yl acetate (**13**, 1.44 g, 6.04 mmol) in 39% overall yield.



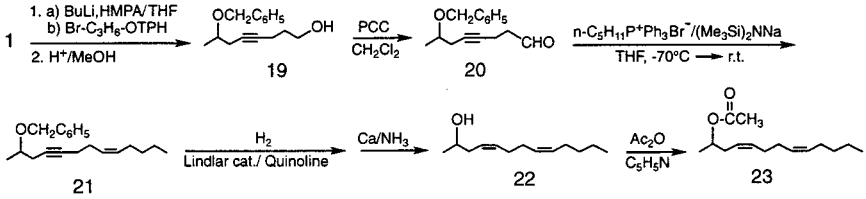
SCHEME 2. Synthesis of (*Z*,*E*)-4,7-tridecadien-2-yl acetate.

*(E,Z)-4,7-Tridecadien-2-yl Acetate (**18**) (Scheme 3).* Compound **9** in THF (5.70 g, 28.8 mmol) (Scheme 1) was extended by one carbon (BuLi at −70°C, then paraformaldehyde, warm to room temperature, and stirring for 24 hr) to the mono-protected diol **14** (95% yield). Reduction of **14** (3.1 g, 13.6 mmol) with lithium aluminum hydride (LAH, 7 molar excess) in refluxing THF produced allylic alcohol **15** (1.90 g, 8.26 mmol) in 60% yield. Metylation of **15** with methanesulfonyl chloride in dichloromethane (0°C) in the presence of 2,4,6-collidine gave 5-(*tert*-butyldimethylsilyloxy)-2-hexenyl methanesulfonate (**16**). Up to 50% formation of the unwanted allylic chloride side product occurred. Reaction of sulfonate **16** with heptynyllithium in the presence of CuI (0.1 equiv.) gave the *tert*-butyldimethylsilyl ether of (*E*)-4-tridecen-7-yn-2-ol (**17**). Removal of the protective group from **17**, hydrogenation, and acetylation (as described for **10** in Scheme 2) afforded after purification (*E*,*Z*)-4,7-tridecadien-2-yl acetate (**18**, 0.18 g, 7.56 mmol) in 9% yield based on **15**.

*(Z,Z)-4,8-Tridecadien-2-ol (**23**) (Scheme 4).* Benzyl ether **1** (4.35 g, 25 mmol) was coupled at −70°C via the lithium salt with 1-*O*-tetrahydropyranyl-3-bromopropanol (1.3 equiv.) [−70°C to room temperature, 24 hr, THF–HMPA (1:1)]. Subsequent removal of the THP group in methanol in the presence of trifluoroacetic acid afforded 7-benzyloxy-4-octyn-1-ol (**19**) (1.32 g, 5.71 mmol). Oxidation of alcohol **19** with PCC in dichloromethane at room temperature, and immediate coupling of the crude aldehyde **20** with *n*-pentylidene triphenylphosphorane in THF yielded the benzyl ether of (*Z*)-8-tridecen-4-yn-2-ol (**21**). Compound **21** partially purified by flash chromatograhy was hydrogenated (4 hr) with Lindlar catalyst in hexanes in the presence of quinoline, and the resulting diene-ether was



SCHEME 3. Synthesis of (*E*,*Z*)-4,7-tridecadien-2-yl acetate.

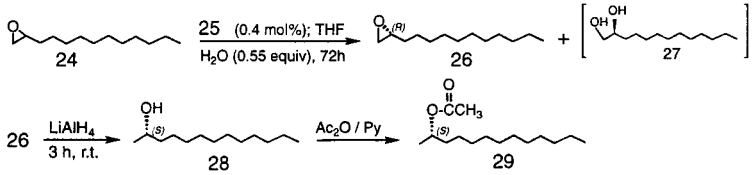


SCHEME 4. Synthesis of (*Z*,*Z*)-4,8-tridecadien-2-yl acetate.

treated with 10 equivalents of calcium in liq. ammonia for 45 min. Usual work-up and purification of the diunsaturated alcohol **22**, followed by overnight acetylation with acetic anhydride in pyridine, yielded (*Z*,*Z*)-4,8-tridecadien-2-yl acetate (**23**, 0.265 g, 1.10 mmol) in 4.5% yield based on **1**.

*(S)- and (R)-2-Tridecanyl Acetate (**29, 34**) (Scheme 5).* Hydrolytic kinetic resolution of racemic 1,2-epoxytridecane (**24**) (1.75 g, 8.80 mmol) with 25 mg of (*R*,*R*)-(−)-salen complex of Co(II) [Jacobsen's catalyst; (*R*,*R*)-(−)-N,N′-bis (3,5-di-*tert*-butylsalicylidene)-1,2-cyclohexanediaminocobalt(II)] (**25**) (Sigma-Aldrich, Oakville, Ontario, Canada) and 90 μl of water (Tokunaga et al., 1997; Schaus et al., 1998; Gries et al., 2000) yielded after 3 d at room temperature (*R*)-1,2-epoxytridecane (**26**) (0.88 g, 4.44 mmol) which was separated from (*S*)-1,2-tridecanediol (**27**) by flash chromatography (hexanes–ether, 10:1). Ring opening of **26** with LAH in ether afforded (*S*)-2-tridecanol (**28**) (0.84 g, 4.24 mmol). Acetylation of **28** gave (*S*)-2-tridecanyl acetate (**29**) (Kamezawa et al., 1994, Sharma et al., 1996) in 47% overall yield and with an enantiomeric excess (ee) of 91.9%, as determined by GC analysis on a Cyclodex-B column. Corresponding hydrokinetic resolution of **24** with the (*S*,*S*)-(+)-salen complex of Co(II), and corresponding follow-up reactions (see above) afforded (*S*)-1,2-epoxytridecane, (*R*)-2-tridecanol, and finally (*R*)-2-tridecanyl acetate (47% overall yield, 91.3% ee).

2-Tridecenyl acetates with *E* and *Z* unsaturation at C-3 to C-12 were synthesized by oxidation (PCC in dichloromethane) of the dodecenols (available in our laboratory from previous studies) and by immediate reaction of the crude aldehydes



SCHEME 5. Synthesis of (*S*)-2-tridecanyl acetate.

with methylmagnesium bromide in ether followed by acetylation (Ac_2O/Py) of the resulting 2-tridecenols.

Absolute Configuration of Pheromone. The absolute configuration of the pheromone was determined in five steps: (1) acetyl lactate derivatization (Slessor et al., 1985) of racemic (*Z*,*Z*)-4,7-tridecadien-2-ol (**5** in Scheme 1); (2) separation of diasteromeric derivatives by HPLC (conditions described above); (3) removal of the acetyl lactate functionality and acetylation of the resulting enantiospecific alcohols; (4) GC-EAD analyses (Figure 3 below) and field testing (Figure 4 below) of (*Z*,*Z*)-4,7-tridecadien-(*S*)-2-yl acetate (2*S*-*Z*4*Z*7) and (*Z*,*Z*)-4,7-tridecadien-(*R*)-2-yl acetate (2*R*-*Z*4*Z*7); and (5) comparison of retention times (Cyclodex-B column) of the saturated equivalents of 2*S*-*Z*4*Z*7 [(*S*)-2-tridecanyl acetate] and 2*R*-*Z*4*Z*7 [(*R*)-2-tridecanyl acetate] with those of enantioselectively synthesized (*S*)- and (*R*)-2-tridecanyl acetate (Scheme 5).

Field Experiments. Field experiments were conducted in Nootka, Mount Newton, and Saanich Douglas-fir seed orchards, and the Douglas-fir seed orchard at the Saanichton Forestry Centre, all near Saanichton, Vancouver Island, British Columbia. Trees were 11–25 years old with spacings within and between rows ranging from 3.8×6.1 m to 4×10 m. At 15–20-m intervals, self-made sticky 2-l milk carton traps (Gray et al., 1984) were suspended from trees 1.5 m above ground in complete randomized blocks. Traps were baited with a gray sleeve stopper (The West Company, Lionville, Pennsylvania, USA) impregnated with test chemicals in HPLC-grade hexane. All test chemicals were >93% chemically and geometrically pure. Enantiomeric excess of 2*S*-*Z*4*Z*7 and 2*R*-*Z*4*Z*7 was 95.5% and 97.8%, respectively, based on GC analyses on a Cyclodex-B column.

Experiment 1 tested candidate pheromone *Z*4*Z*7 at doses of 1, 10, 100, and 1,000 μg. Experiments 2 and 3 tested *S* and *R* enantiomers of *Z*4*Z*7 singly and in combination at doses of 10 and 2 μg, respectively. Experiment 4 tested attractiveness of racemic *Z*4*Z*7 at 50 μg versus that of caged female *C. oregonensis*.

Despite transformation, trap catch data were not normally distributed and were, thus, analyzed by nonparametric analyses of variance (Friedman's test) followed by comparison of means by Bonferroni (Dunn) t test (Zar, 1984; SAS/STAT, 1988). In all analyses, $\alpha = 0.05$.

RESULTS AND DISCUSSION

Pheromone Analyses. Comparison of GC traces of abdominal tip extracts of male and female *C. oregonensis* failed to disclose sex-specific volatiles. Coupled GC-EAD analyses of female pheromone extract revealed a single EAD-active compound (**A** in Figure 2) that occurred below detection threshold of the flame ionization detector of the GC. Retention indices (RI) (Van den Dool and Kratz, 1963) of **A** (DB-5: 1605; DB-210: 1872; DB-23: 1932; SP-1000: 1927;

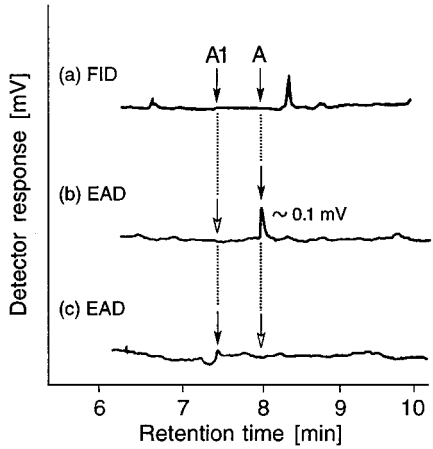


FIG. 2. Representative GC-EAD recording (8 replicates) of pheromone gland extract of female *C. oregonensis*; (**a**) flame ionization detector (FID) chromatograph of 10 female equivalents (10 FE) of pheromone gland extract; (**b, c**) corresponding recording of electroantennographic detector (EAD: male *C. oregonensis* antenna) responses to 10 FE of (**b**) untreated and (**c**) hydrogenated pheromone extract. Chromatography: Hewlett Packard 5890A gas chromatograph; splitless injection; injector temperature: 220°C; detector temperature: 250°C; DB-23 column; temperature program: 100°C (1 min) 10°C per min to 200°C. A = (*Z*,*Z*)-4,7-tridecadien-2-yl acetate; A1 = tridecan-2-yl acetate.

Cyclodex B: 1690), and RI intercolumn differentials, suggested that it had an ester (acetate) functionality. GC-EAD analysis of hydrogenated pheromone extract revealed a new EAD-active compound (**A1** in Figure 2), suggesting that hydrogenation of **A** had resulted in a saturated volatile (**A1**) with different chromatographic characteristics [RIs: 1631 (DB-5); 1890 (DB-210); 1879 (DB-23); and 1870 (SP-1000)]. Compound **A1** was determined to be 2-tridecanyl acetate [an aggregation pheromone component of *Drosophila mulleri* and *D. busckii* (Kamezawa et al. 1994)], which cochromatographed with **A1** on all above-referenced columns and had comparable EAD activity as **A1**. Similar chromatographic characteristics of **A** and **A1** suggested that the former was a tridecen-2-yl acetate. However, syntheses and chromatography of all possible tridecen-2-yl acetates determined that neither co-chromatographed with **A**, nor elicited antennal responses indicative of a potential double bond position.

Hypothesizing then that **A** was a nonconjugated tridecadien-2-yl acetate, candidate tridecadien-2-yl acetates were approximated (Gries et al., 1993) by (1) determining retention index differentials between tridecan-2-yl acetate and each of the tridecen-2-yl acetates (Table 1), and (2) adding these differentials to, or subtracting them from, the respective tridecen-2-yl acetate. For example, the RI 1604

TABLE 1. RETENTION INDICES AND DIFFERENTIALS (BOLD FACE TYPE) BETWEEN SYNTHETIC TRIDECEN-2-YL ACETATES AND SYNTHETIC TRIDECAN-2-YL ACETATE (SAT) ON GC COLUMNS COATED WITH DB-5, DB-210, DB-23, OR SP-1000

dbp[a]	DB-5	DB-210	DB-23	SP-1000
sat	1631	1890	1879	1870
E 3	1620: −**11**	1864: −**26**	1890:+11	1900: +**30**
E 4	1616: −**15**	1876: −**14**	1885:+**6**	1884: +**14**
E 5	1614: −**17**	1859: −**31**	1885:+**6**	1891: +**21**
E 6	1614: −**17**	1871: −**19**	1891:+**12**	1894: +**24**
E 7	1618: −**13**	1881: −**9**	1897: +**18**	1894: +**24**
E 8	1619: −**12**	1875: −**15**	1899:+**20**	1900: +**30**
E 9	1621: −**10**	1881: −**9**	1901:+**22**	1900: +**30**
E 10	1627: −**4**	1889: −**1**	1910:+**31**	1910: +**40**
E 11	1635: +**4**	1895: +**5**	1931:+**52**	1931: +**61**
Δ12	1623: −**8**	1904: +**14**	1931: +**52**	1920: +**50**
Z 3	1589: −**42**	1796: −**94**	1830: −**49**	1844: −**26**
Z 4	1617: −**14**	1871: −**19**	1898: +**19**	1894: +**24**
Z 5	1604: −**27**	1871: −**19**	1888: +**9**	1880: +**10**
Z 6	1609: −**22**	1873: −**17**	1896: +**17**	1890: +**20**
Z 7	1610: −**21**	1881: −**9**	1903: +**24**	1894: +**24**
Z 8	1618: −**13**	1890: ±**0**	1913: +**34**	1903: +**33**
Z 9	1624: −**8**	1898: +**8**	1920: +**41**	1911: +**41**
Z 10	1630: −**1**	1903: +**13**	1929: +**50**	1920: +**50**
Z 11	1648: +**17**	1925: +**35**	1956: +**77**	1951: +**81**

[a] dbp = double bond position.

of (*E*,*E*)-4,8-tridecadien-2-yl acetate on DB-5 was predicted by subtracting the RI differential between tridecan-2-yl acetate and (*E*)-8-tridecen-2-yl acetate (=12; Table 1) from the RI of (*E*)-4-tridecen-2-yl acetate (1616 − 12 = 1604). Taking the data for all five columns into account, (*Z*,*Z*)-4,8-tridecadien-2-yl acetate (*Z*4*Z*8) was predicted to be the most probable diene acetate to match chromatographic characteristics of **A**. However, synthetic *Z*4*Z*8 (Scheme 4) had RIs 1-3 RI units different from those of the target **A** on all five columns.

Strong EAD activity of *Z*4*Z*8 suggested that its molecular structure was similar to that of the candidate pheromone **A**. An allylic double bond in C-3, instead of C-4, would have greatly decreased the RI of the molecule (RG + GG, unpublished data) and was discounted. Hypothesizing then that the geometry and position of the double bond in C-8 was incorrect, (*Z*,*Z*)-4,7-tridecadien-2-yl acetate (*Z*4*Z*7) was synthesized (Scheme 1) as an alternative structure for the candidate pheromone. This compound, but none of its three isomers (*ZE*, *EZ*, *EE*) (Schemes 1–3), cochromatographed with **A** on all columns, and elicited comparable antennal responses.

In GC-EAD analyses that separated the enantiomers of *Z*4*Z*7 with baseline resolution (Figure 3, a), antennal responses were obtained only to the early

eluting enantiomer in a racemic standard (Figure 3, b) or in pheromone extract (Figure 3, c). This enantiomer was determined to have the *S* configuration, because its hydrogenated equivalent had a retention time on Cyclodex-B that was identical with that of enantiospecifically synthesized (*S*)-2-tridecanyl acetate (**29**) (Scheme 5). (*R*)-2-Tridecanyl acetate, in contrast, eluted 0.35 min later. Lack of response to later eluting 2*R*-*Z*4*Z*7 could not be attributed to a refractory period of the antenna, because two sequential injections (at 0.5 min intervals) of isothermally chromatographed 2*S*-*Z*4*Z*7 elicited two very similar antennal responses (Figure 3, d). When 2*S*- and 2*R*-*Z*4*Z*7 were analyzed individually, only the *S* enantiomer was EAD-active, supporting contention that female *C. oregonensis* produce 2*S*-*Z*4*Z*7 for pheromonal attraction of mates.

Field Experiments. In experiment 1, a lure dose of 100 μg of racemic Z4Z7 was more effective than lower or higher lure doses in attracting male *C. oregonensis* (Figure 4, experiment 1), indicating that proper lure loadings are critical for optimal attractiveness. Captures of male *C. oregonensis* in traps baited with 2*S*-*Z*4*Z*7 (Figure 4, experiments 2 and 3) were consistent with EAD-activity only of the *S* enantiomer (Figure 3, b). The few males captured in traps baited with 2*R*-*Z*4*Z*7 (Figure 4, experiment 2) were probably attracted to trace amounts of the *S* enantiomer in the bait. The antipode 2*R*-*Z*4*Z*7 is not part of the *C. oregonensis* pheromone because it elicited neither EAD-activity (Figure 3, b) nor behavioral activity when tested alone at a low dose (Figure 4, experiment 3) or in combination with pheromonal 2*S*-Z4Z7 (Figure 4, experiments 2 and 3). Similar numbers of males captured in traps baited with synthetic *Z*4*Z*7 (50 μg) or virgin female *C. oregonensis* (Figure 4, experiment 4) indicate competitive attractiveness of natural and synthetic pheromone.

Pheromones in Cecidomyiids and Practical Implications. Evidence for female-produced sex pheromones has been reported for 10 cecidomyiids (reviewed by Harris and Foster, 1999) but only four pheromones have been identified. In pioneering studies, (*E*)-10-tridecen-(*S*)-2-yl acetate was determined to be a sex pheromone component of the Hessian fly, *Mayetiola destructor* (Foster et al., 1991; Harris and Foster, 1991; Millar et al., 1991). Attractiveness of this compound to male *M. destructor* in wind-tunnel but not field experiments suggested that female *M. destructor* deploy additional pheromone components that have not yet been identified (Harris and Foster, 1999). Meticulous work by Hillbur et al. (1999, 2000, 2001) deciphered the three-component pheromone of the pea midge, *Contarinia pisi*. Synthetic (2*S*,11*S*)-diacetoxytridecane, (2*S*,12*S*)-diacetoxytridecane, and 2-acetoxytridecane at a blend ratio as found in pheromone gland extracts attracted male *C. pisi* in both wind-tunnel and field experiments. Concurrent work by Gries et al. (2000) revealed the pheromone of the orange wheat blossom midge, *Sitodiplosis mosellana*, to be (2*S*,7*S*)-nonanediyl dibutyrate, which attracted male *S. mosellana* in field experiments in Saskatchewan. While *SR*, *RS*,

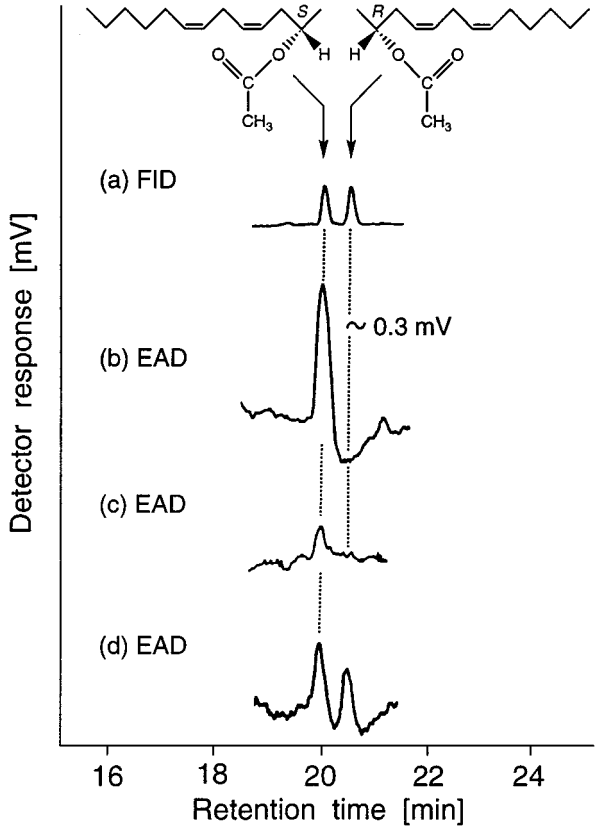


FIG. 3. Representative GC-EAD recording (5 replicates) of synthetic standards and pheromone gland extract of female *C. oregonensis*; (**a**) flame ionization detector (FID) chromatogram of synthetic (*Z*,*Z*)-4,7-tridecadien-2-yl acetate (*Z*4*Z*7) with baseline resolution of the enantiomers (*Z*,*Z*)-4,7-tridecadien-(*S*)-2-yl acetate (2*S*-*Z*4*Z*7; left) and (*Z*,*Z*)-4,7-tridecadien-(*R*)-2-yl acetate (2*R*-*Z*4*Z*7; right); (**b–d**) corresponding recordings of electroantennographic detector (EAD: male *C. oregonensis* antenna) responses to (**b**) synthetic *Z*4*Z*7, (**c**) 10 female equivalents of pheromone gland extract and (**d**) two sequential injections of 2*S*-*Z*4*Z*7. Note: first and second injection of 2*S*-*Z*4*Z*7 were separated by 0.5 min., equivalent to the 0.5 min separation of 2*S*-*Z*4*Z*7 and 2*R*-*Z*4*Z*7 in the racemic standard (**a**); lack of response to later eluting 2*R*-*Z*4*Z*7 (**b**) could not be attributed to a refractory period of the antenna, because two sequential injections (at 0.5-min intervals) of isothermally chromatographed 2*S*-*Z*4*Z*7 elicited two very similar antennal responses (**d**). Chromatography: Hewlett-Packard 5890A gas chromatograph equipped with a chiral Cyclodex-B column; temperature program: 150°C isothermal; split injection of 25 ng per compound with 0.5 ng reaching the EAD detector.

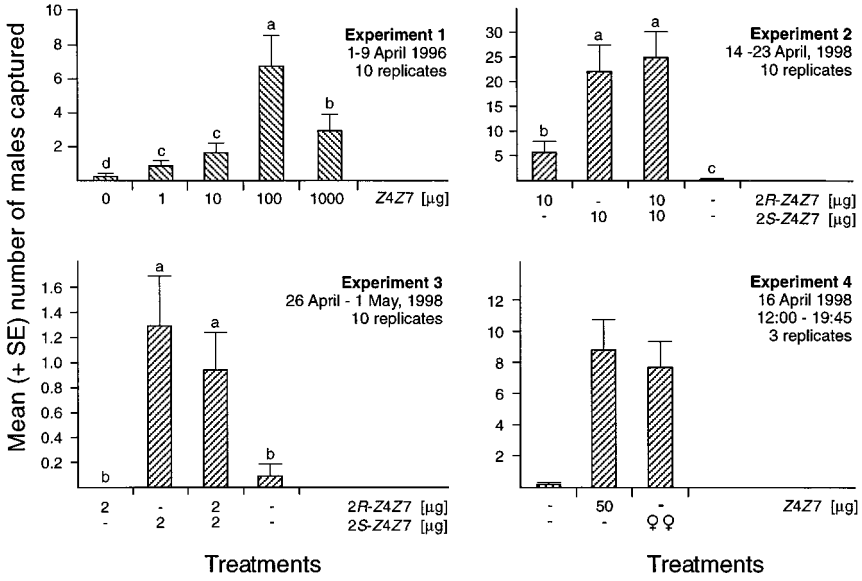


FIG. 4. Mean (+SE) number of male *C. oregonensis* captured during experimental periods in sticky Delta milk carton traps baited with various doses of racemic (*Z*,*Z*)-4,7-tridecadien-2-yl acetate (*Z*4*Z*7) (experiment 1), (*Z*,*Z*)-4,7-tridecadien-(*S*)-2-yl acetate (2*S*-*Z*4*Z*7), and (*Z*,*Z*)-4,7-tridecadien-(*R*)-2-yl acetate (2*R*-*Z*4*Z*7) singly and in combination (experiments 2, 3), or baited with racemic *Z*4*Z*7 or caged virgin female *C. oregonensis* (experiment 4). Douglas-fir seed orchards near Saanichton, Vancouver Island, British Columbia. Bars in each experiment with different letter superscripts are significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means [Bonferroni (Dunn) *t* test, $P < 0.05$] (SAS/STAT, 1988).

and *RR* stereoisomers of the pheromone had no adverse effects on attraction of male *S. mosellana*, attraction of male *C. pisi* to the synthetic *C. pisi* pheromone blend (see above) was strongly inhibited by the presence (20%) of the 2*S*,11*R*- and 2*R*,11*S*-stereoisomers of diacetoxytridecane (Hillbur et al., 2001). Male *C. oregonensis*, in contrast, did not perceive the antipode of pheromonal 2*S*-*Z*4*Z*7 (Figure 3, b) and were not inhibited by it (Figure 4, experiments 2 and 3).

The identification of the *C. oregonensis* pheromone should lead to improved monitoring of *C. oregonensis* populations and management decisions in commercial seed orchards. In both 2000 and 2001, numbers of male *C. oregonensis* captured in pheromone-baited traps in seed orchards in Washington and Oregon were correlated with numbers of both egg-infested scales in conelets and damaged seed in cones (Morewood et al., 2002). Trap captures of male *C. oregonensis* are, thus, predictive of seed damage at harvest, and should help orchard managers decide whether insecticidal control of *C. oregonensis* populations is warranted.

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QUANTITATIVE GC ANALYSIS OF SECONDARY ALCOHOL PHEROMONES: DETERMINATION OF RELEASE RATE OF RED PALM WEEVIL, *Rhynchophorus ferrugineus*, PHEROMONE FROM LURES

A. ZADA,[1,*] V. SOROKER,[1] M. HAREL,[1] J. NAKACHE,[2] and E. DUNKELBLUM[1]

[1]*Institute of Plant Protection*
The Volcani Center
Bet-Dagan, 50250, Israel

[2]*Eden Experiment Station*
Bet Shean, Israel

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Abstract—Aliphatic secondary alcohols are components of several aggregation pheromones of important beetle and weevil pests. Some of these pheromones are used frequently for the monitoring and mass trapping of the relevant insects. We encountered severe difficulties in direct GC quantitative analysis of these compounds. Therefore, we developed a simple GC analysis of secondary alcohols converting them to trifluoroacetyl derivatives and using secondary alcohol acetates as internal standards. This method was applied for the quantitative analysis of several secondary alcohols, including the aggregation pheromone components of the almond bark beetle and the red palm weevil. The release rate of the latter pheromone from commercial lures was also determined.

Key Words—Aggregation pheromones, secondary alcohols, trifluoroacetyl derivatives, GC analysis.

INTRODUCTION

Several aliphatic secondary alcohols are common components of aggregation pheromones of weevils and beetles (Bartelt, 1999; Schlyter and Birgersson, 1999). The use of pheromone lures in pest management requires a quantitative determination of the content in order to measure the release rate of the pheromone and the longevity of the lures. Capillary GC analysis, with an internal standard, is the best

* To whom correspondence should be addressed. E-mail: anatzada@volcani.agri.gov.il

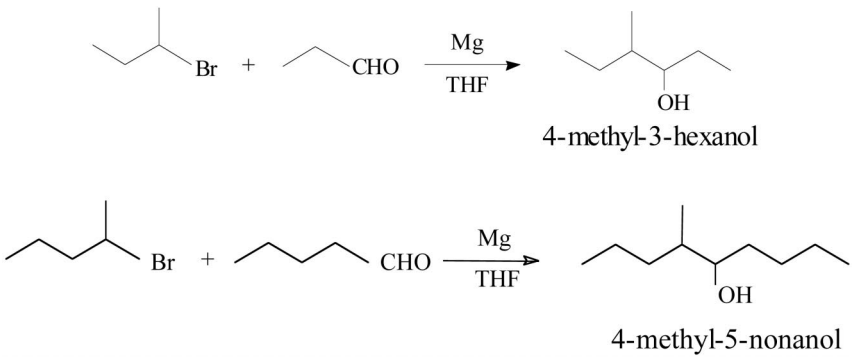
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method for quantitation. However, peaks of secondary alcohols have a tendency to broaden and tail on different capillary columns, which makes their direct analysis difficult and inaccurate. The problem is particularly severe when analyses are performed repeatedly on the same column.

In this paper, we present a simple GC method of quantitation of secondary alcohols using their trifluoroacetyl derivatives and appropriate acetates as internal standards. The effect of the mode of injection on the analysis has also been studied. The following alcohols were analyzed: 4-methyl-5-nonanol, 4-methyl-3-heptanol, 4-methyl-3-hexanol, and 2-octanol. These alcohols (except 2-octanol) are pheromone components of two agricultural pest insects, the red palm weevil (*Rhynchophorus ferrugineus*) (Perez et al., 1996) and the almond bark beetle (*Scolytus amygdali*) (Ben-Yehuda et al., 2000), respectively.

METHODS AND MATERIALS

Chemicals. Mixtures of syn/anti 4-methyl-3-hexanol and 4-methyl-5-nonanol were prepared by Grignard reaction (Scheme 1) from 2-bromobutane and propionaldehyde and from 2-bromopentane and valeraldehyde, respectively, using magnesium turnings in THF (dried over Na + benzophenone). Distillation was carried out under reduced pressure (142°C/760 mmHg and 87°C/12 mmHg, respectively) to obtain the pure alcohols (97–100% by GC). The acetate derivatives were prepared from the corresponding alcohols and acetic anhydride in pyridine and purified by chromatography on SiO_2, using hexane and 1% ether as eluent. All other chemicals, including syn/anti (60:40) 4-methyl-3-heptanol, were purchased from Aldrich.



SCHEME 1.

Gas Chromatography. Analyses were performed on an HP6890 gas chromatograph equipped with an HP5 column (30 m × 0.25 mm ID; 0.25 μm), flame

ionization detector, and a split/splitless injector. Helium was used as the carrier gas with a flow rate of 1.5 ml/min. The analysis was performed in the split mode with a ratio of 10:1. The column was kept at 50°C for 3 min and then programmed at a rate of 20°C/min to 100°C for the analysis of 4-methyl-3-hexanol, 4-methyl-3-heptanol, and 2-octanol and kept at 60°C for 2 min, and then programmed at a rate of 20°C/min to 130°C for the analysis of 4-methyl-5-nonanol. The injector and detector were maintained at 220°C.

Procedure of Analyses. Solutions of 4-methyl-5-nonanol, 4-methyl-3-heptanol, 4-methyl-3-hexanol, and 2-octanol were prepared in hexane at a concentration of 1 mg/ml ($= 1\ \mu g/\mu l$). Representative aliquots of each solution (1, 5, 10, 50, 75, 100, 150, and 200 μl) were placed in a 4-ml vial containing 1 ml of hexane. Then, an appropriate amount of trifluoroacetic anhydride (50–100 μl) was added, and the vial was shaken thoroughly. The reaction mixture was shaken occasionally for 1 hr at room temperature and then cooled in an ice-bath. The derivatization was terminated by addition of 1 ml of 5% NH_4OH (aq) and 100 μl of a hexane solution containing 100 μg of the appropriate acetate derivative (stock solution of 1 mg/ml). Fifty micrograms of the acetates' internal standard was used for the small amounts of analyzed alcohols (1–10 μg). The mixture was shaken thoroughly, and the organic phase was separated with a pipet. Aliquots of approximately 2 μl of the organic phase were injected (three replicates) into the gas chromatograph. A representative analysis of 100 μg of 4-methyl-5-nonanol with 50, 75, and 100 μg of the corresponding acetate as internal standard was injected several times and gave the same results.

Application of the Method with Red Palm Weevil Pheromone Lures. Pheromone lures of the red palm weevil were purchased from ChemTica (Costa Rica). They contained 750 ± 5 mg pheromone of which 90% is 4-methyl-5-nonanol and 10% is 4-methyl-5-nonanone enclosed in a plastic bag with a membrane (Hallett et al., 1999). The lures were placed in weevil bucket traps in a date plantation in the Eden ranch, Jordan Valley. The traps also contained a mixture of plant material (molasses sugar and dates) in water and a bottle of ethyl acetate. Every 10 d, three lures were removed from the orchard and kept at 4°C until analyzed. The plastic bag containing the pheromone was cut into pieces and immersed in 20 ml of dichloromethane (CH_2Cl_2) to extract the pheromone. The samples were kept in an ultrasound bath for 30 min and left overnight at 4°C. The plastic material was not affected by the solvent. For GC analysis, samples were diluted by a factor of 100. Then, an aliquot of 100 μl was further diluted with 1 ml hexane and 50 μl of the appropriate acetate standard were added.

RESULTS AND DISCUSSION

It is well known that quantitative GC analysis of secondary alcohols is difficult and inaccurate due to peak broadening and tailing (Krupcik et al., 1975).

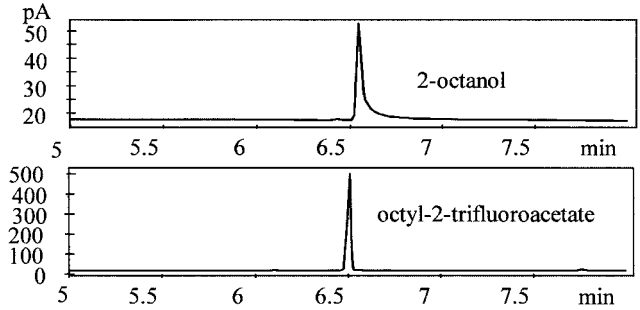


FIG. 1. GC profiles of 2-octanol versus octyl-2-trifluoroacetate. HP5 column was kept at 50°C for 3 min and then programmed at a rate of 20°/min to 100°C. Split ratio 10:1.

Despite the availability of improved capillary GC columns today, secondary alcohols display erratic behavior, particularly with aging columns. The columns are too expensive to be replaced after a short usage. Periodical weighing has been recommended as an alternative method in order to determine release rates of such pheromones from lures (Vickers et al., 1998; A.C. Oehschlager, personal communication). However, loss of weight is not specific to the loss of the pheromone alone. In addition, it gives erratic results due to absorption of dust and moisture. GC–FID analysis, with an appropriate internal standard, is the most accurate method, provided that peaks are sharp and the analysis is consistent.

During our work on mass trapping of the red palm weevil and the almond bark beetle with pheromone lures, we were in need of a reliable quantitative analysis of their respective main pheromone components, 4-methyl-5-nonanol and 4-methyl-3-heptanol. Direct GC analysis of the alcohol gave inaccurate results and, therefore, we looked for an alternative route and decided to use a derivatization method. We chose trifluoroacetylation because this derivatization of alcohols is easy to perform. It is quantitative and the formed trifluoroacetates can be readily analyzed by capillary GC-FID (Krupcik et al., 1994). Peaks are symmetrical and sharp (Figure 1). Therefore, sensitivity is higher as compared with the parent alcohols. The method has been employed for the analysis of alcohols and sugars (Krupcik et al., 1994; Hisatomi et al., 2000). However, as far as we know, trifluoroacetylation has not been used for quantitative analysis of secondary alcohols by GC. The FID response of the acetates and the trifluoroacetates is practically identical (GC-FID factor >0.98), and the latter always elute before their corresponding acetates (Representative example is in Scheme 2 and Figure 2).



4-methyl-3-heptyltrifluoroacetate (Analyte)

4-methyl-3-heptylacetate (Internal standard)

SCHEME 2.

The method was tested with various amounts of 4-methyl-3-hexanol, 4-methyl-3-heptanol, 2-octanol, and 4-methyl-5-nonanol in the range of 1–200 μg. Representative results of 4-methyl-3-heptanol and 4-methyl-5-nonanol, presented in Figures 3 and 4, indicate an excellent correlation (>99%) between the amount of secondary alcohol inserted and detected by the GC method. The use of the acetate of the same alcohol as the internal standard is not essential. For example, 4-methyl-3-hexanol and 2-octanol were analyzed with 4-methyl-3-heptylacetate as the internal standard, and the results were accurate. For better peak shape and resolution, we recommend performing the GC analysis in the split mode and programing the oven temperature so that both the analyte and the standard will be analyzed isothermally. The trifluoroacetylation reaction is quantitative, and no

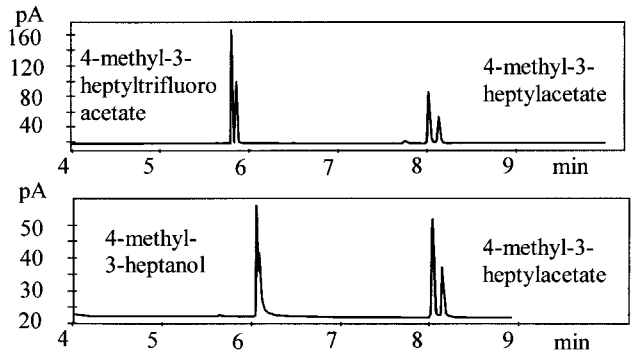


FIG. 2. GC profiles of the analysis of syn/anti (60:40) 4-methyl-3-heptanol with its acetate derivative as internal standard versus analysis of 4-methyl-3-heptyltrifluoroacetate using the same standard. HP5 column was kept at 50°C for 3 min and then programmed at a rate of 20°/min to 100°C. Split ratio 10:1

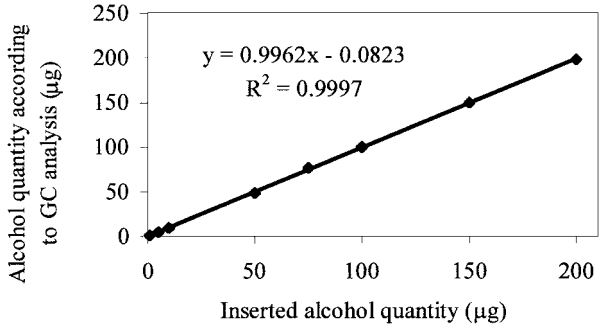


FIG. 3. GC quantitative analysis of 4-methyl-3-heptanol. Each point is the average of three anlyses.

residual alcohol was detected by GC in all cases. The use of varying amounts of alcohols or internal standard does not introduce any significant error, as shown by the excellent reproducibility.

The red palm weevil, *Rhynchophorus ferrugineus* (RPW), is a serious pest of dates. The major component of its aggregation pheromone is (*S*,*S*)-4-methyl-5-nonanol (Perez et al., 1996). Commercial lures, containing racemic syn/anti 4-methyl-5-nonanol, are being used in the Far and Middle East for mass trapping (Hallett et al., 1999; Vidyasagar et al., 2000). The determination of the release rate of the pheromone is essential in order to evaluate the longevity of the lures. Direct analysis of 4-methyl-5-nonanol produced erratic results. We applied our method

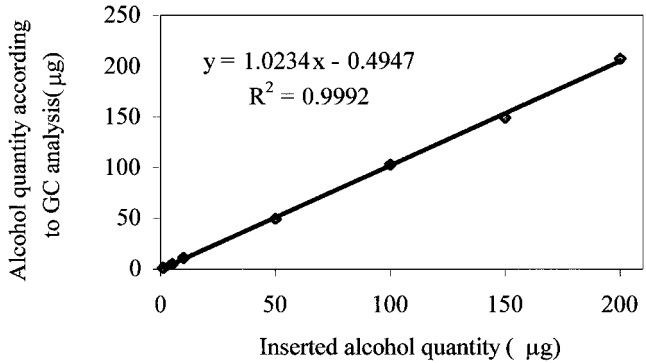


FIG. 4. GC quantitative analysis of 4-methyl-5-nonanol. Each point is the average of three anlyses.

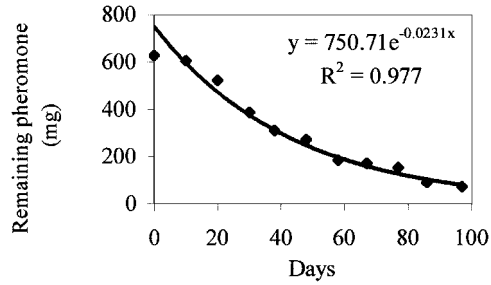


FIG. 5. Release rate of 4-methyl-5-nonanol from the ChemTica lures under field conditions.

and obtained consistent results. The behavior of the lure for the whole period can be represented by an exponential curve (Figure 5). However, the release rate of about 8 mg/day is linear for 58 days, and then the release rate decreases to 3–4 mg/day for another 40 days (Figures 5 and 6). This behavior corresponds with the manufacturer's specifications (release rate of 3–10 mg/day) and the temperature dependence of the release rate. The average temperatures in the plantation for the first period (0–58 d) of the field test was 23.5–36.6°C, and of the second one (58–98 d) was 17–29.8°C.

In conclusion, the quantitation of secondary alcohol aggregation pheromones is of practical importance and is not merely an analytical exercise. Several beetle and weevil pheromones containing such components are being used for mass trapping in Integrated Post Management programs. The method presented in this study will facilitate the evaluation of such lures and will enable the determination of their longevity and release rate profiles.

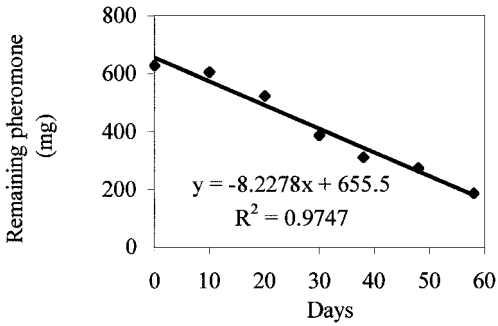


FIG. 6. Release rate of 4-methyl-5-nonanol from the ChemTica lures for the first period of 58 d of the field test.

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FOOD EXPERIENCE ON THE PREDATORY BEHAVIOR OF THE ANT *Myrmica rubra* TOWARDS A SPECIALIST MOTH, *Acrolepiopsis assectella*

A. M. LE ROUX, G. LE ROUX, and E. THIBOUT

Institut de Recherche sur la Biologie de l'Insecte
UMR CNRS 6035
Faculté des Science
Parc Grandmont, 37200 Tours, France

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Abstract—Entomophagous insects are often repelled by the secondary compounds of the plants eaten by their prey. These compounds, therefore, take on a defensive role for the phytophagous species that sequester them. Given that numerous entomophagous species are capable of learning, the effects on the foraging behavior of a repeated experience were investigated in the predatory ant *Myrmica rubra*. The sulfur amino acids methyl-cysteine sulfoxide (MCSO) and propyl-cysteine sulfoxide (PCSO) produced by *Allium* plants were identified in caterpillars of the leek moth *Acrolepiopsis assectella*. Three behavioral studies were carried out, with or without prior familiarization with caterpillars reared either on leek or on an artificial diet containing no *Allium* compounds. In choice tests with the two types of caterpillars, unfamiliarized ants displayed a preference for caterpillars reared on the artificial diet, but this preference disappeared or was reversed in both young and old ants after familiarization.

Key Words—Foraging experience, predation, ants, leek moth, prey selection, learning, *Allium*, sulfur compounds, habituation.

INTRODUCTION

Plant secondary compounds found in varying quantities in prey often lead to rejection by generalist predators. The secondary compounds, thus, have a protective role for specialist phytophagous insects (Bernays, 1989; Bernays and Cornelius, 1989; Dyer, 1995). Among the factors that can modulate this defense phenomenon are the secondary compound concentration in the plant (Bjorkman and Larsson,

* To whom correspondence should be addressed. E-mail: thibout@univ-tours.fr

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1991; Hare and Eisner, 1993), the morphology of the prey (Dyer, 1995) and its defensive behavior (Bernays and Cornelius, 1989; Bjorkman and Larsson, 1991), the abundance of predators and their ability to locate the prey (Camara, 1997), or experience and learning.

The involvement of experience acquired by the predator during foraging, and observed in the bug *Podisus maculiventris* (Traugott and Stamp, 1996), has frequently been studied in ants (Dejean, 1988; Dejean et al., 1990; Fourcassié and Traniello, 1993; Hare and Eisner, 1993; Schumann and Buschinger, 1994; Dyer and Bowers, 1996). Similarly, learning seems to play a role in the predatory behavior of ants that are offered caterpillars of the leek moth, *Acrolepiopsis assectella* (Lepidoptera: Yponomeutoidea), a specialist phytophagous species of *Allium* plants (Nowbahari and Thibout, 1992). Caterpillars of the leek moth feed on the leek, *Allium porrum*, a plant that produces nonprotein sulfur amino acids, S-alk(en)yl-cysteine sulfoxides (Boscher et al., 1995) that are precursors of sulfur volatiles (Férary et al., 1998). These caterpillars are attacked markedly less often by *Formica selysi* and *Formica fusca* than those fed on an artificial diet with either little or none of these compounds (Nowbahari and Thibout, 1992).

This work addresses the following questions: Do leek moth caterpillars sequester sulfur compounds? Is it possible to generalize the dietary preference to other ant species? Can the consumption of novel or repellent prey occur as a result of familiarization during the life cycle of the predator?

METHODS AND MATERIALS

The Phytophagous Insect. The caterpillars came from two rearings. The first was carried out on the host plant, the leek (CL: caterpillars on leek), under the following conditions: 16L:8D, synchronous temperature 25°C:18°C, and 70% ± 10% relative humidity. This strain was renewed each year by caterpillars collected in leek fields around Tours, France. The second rearing was maintained for several years in the laboratory on an artificial diet containing 10% of freeze-dried leek leaf powder (Arnault, 1979), on 12L:12D, constant temperature 25°C and 70% ± 10% relative humidity. Eggs were taken from this rearing, and neonatal caterpillars were reared on an artificial diet containing neither leek powder nor leek in order to obtain caterpillars that had had no contact with leek compounds (CD: caterpillars on diet). The CL and CD used at the fifth instar, after sexual differentiation, were killed by freezing at −18°C in order to avoid escape and defensive behavior, before being introduced into the experimental device.

The Predator. The red ant *Myrmica rubra* (Hymenoptera: Formicidae), which has the ability to learn (Le Roux et al., 1997), is common in the Touraine region of France and is an omnivorous polygynous species that builds colonies of several thousand workers (Elmes and Keller, 1993). It lives in open environments such

as prairies, gardens, and fairly damp wastelands and can be a predator of the leek moth. Every year, colonies were taken from the Loire riverbanks near Montlouis at the beginning of September. They were split into several groups (15–30 workers) and maintained at 12L:12D and 24°C. Each group was isolated in a nest consisting of a 15-cm-long × 1.5-cm-diam glass tube in a sleeve of black paper with a water holder at one end. The other end of the nest opened into a hunting area formed by a 5-cm-high × 8-cm-diam. cylindrical box. Twice a week, the ants received half a larva of *Tenebrio molitor* and a drop of honey in the hunting area. During the experiments, *T. molitor* and honey were replaced by two leek moth caterpillars 2 cm away from the junction between the tube and the collecting area, and 1 cm apart.

Analysis of Sulfur Amino Acid Content of Caterpillars. The two major sulfur amino acids produced by *A. porrum* are methyl-cysteine sulfoxide (MCSO) and propyl-cysteine sulfoxide (PCSO) (Freeman and Whenham, 1975). PCSO has been shown to be a repellent for formicines (Nowbahari and Thibout, 1992). The amounts of these compounds were measured in the preimaginal stages of the leek moth. Two CD taken from the diet, 2 CL taken from the leek, 2 CL starved for 5 hr, 2 chrysalids from CL, 40 exuviae from CL, and finally a 10-mg piece of green leek leaf (weight of a caterpillar is approx. 10 mg, of a chrysalis approx. 7 mg, and of an exuvia approx. 0.05 mg) were crushed in methanol. They were centrifuged for 30 min at −4°C at 6000 rpm, and the supernatant was retained. The residue obtained after evaporation of the methanol was diluted in trichloracetic acid at 6% in order to precipitate the proteins and to eliminate them by centrifugation. Amino acids were determined using derivatization coupled with reverse-phase HPLC. The Waters Pico-Tag method (Bidlingmeyer et al., 1984) used phenylisothiocyanate as a derivatization reagent (Auger et al., 1993). The HPLC system used was a Waters chromatograph consisting of a two-pump delivery system and a UV detector with a wavelength fixed at 254 nm. The column was a Waters Novapack C18 (3.9 × 300 mm). The solvent system consisted of two Waters eluents. The addition of controlled quantities of alkyl-cysteine sulfoxides synthesized in the laboratory (Auger and Thibout, 1981) allowed a quantitative study to be carried out by comparing retention time and peak areas. These operations were repeated at least 4 times (Table 1).

Experiments. All experiments were carried out under choice conditions in the ant-rearing room. An experiment began when 1 CL and 1 CD, or 2 CL, were simultaneously placed in the hunting area. A full choice test comprised five consecutive presentations of two caterpillars at 2-d intervals (D1, D3, D5, D7, and D9). Twenty-four hours after introduction, the number of caterpillars of each category taken into the nest was recorded, and their state of consumption was noted as follows: not consumed, partially consumed, or totally consumed.

Preliminary Behavioral Experiment. The ability to distinguish easily between males and females (visible testicles in the male caterpillar) was used as

TABLE 1. CONCENTRATIONS OF SULFUR AMINO ACIDS OF DIVERSE LEEK MOTH INSTARS FROM TWO REARINGS[a]

Instar	*N*	MCSO	PCSO
CD	4	0	0
CL	6	0.9 (0.3–2.7)	3.7 (0.7–5.6)
CL St.	5	t	2.1 (0.8–6.8)
NL	8	0.1 (0–1)	0.9 (0–2.0)
EL	5	7.2 (0-35.8)	62.9 (10.3–137.5)
LL	4	1.5 (0-5.9)	39.1 (13.3–70.7)

[a] Leek leaves are used as control. Values are mean (range). CD: fifth instar caterpillars reared on diet without leek; CL: fifth instar caterpillars reared on leek; CL St.: CL starved for five hour; NL: nymphs (chrysalid) from leek rearing; EL: exuviae from leek rearing; LL: Leek leaves; t: traces; *N*: number of repetitions.

a method of avoiding confusion between CL and CD, i.e., by alternately placing the CL of one sex with the CD of the other sex. In addition, the right–left placing of the caterpillars was also alternated in order to eliminate a potential effect of environmental dissymmetry. Ten groups of 30 workers were tested with some CL in order to check that *M. rubra* behaved in the same way towards males and females.

Behavior of Ants. A first experiment allowed 10 groups of 30 workers that had never been placed previously with leek moth caterpillars (unfamiliarized) to choose between CL and CD. Moreover, ten groups of 15 young workers were fed for 30 days on 2 CL every 2 d (young familiarized). A choice test was then carried out between CL and CD. Subsequent to the behavioral modifications observed after familiarization in young workers, a comparable experiment was done using old workers collected during the previous year and kept in the laboratory for 6 months. Thirty days before the choice test, the 10 groups of 20 old workers (old familiarized) were fed every 2 d with 2 CL in the same way as the young workers. The number of caterpillars taken back to the nest and their state of consumption were noted. The data were compared using a χ^2 contingency test.

RESULTS

Amount of Sulfur Amino Acids in Caterpillars. The sulfur amino acids MCSO and PCSO synthesized by the leek were not detected in the CD (Table 1). The two compounds were generally found in the preimaginal stages of CL. The CL only just taken from the leek were richer in sulfur amino acids than those starved for several hours. The chrysalids arising from rearing on leek were low in sulfur amino acids, whereas the corresponding exuviae were rich. The concentrations of sulfur amino acids measured in the leek moth do not reflect exactly the concentrations

TABLE 2. LEEK MOTH CATERPILLARS FROM TWO REARINGS CONSUMED IN 24 HR BY UNFAMILIARIZED OR FAMILIARIZED (YOUNG AND OLD) *Myrmica rubra* WORKERS, ACCORDING TO DIET OF CATERPILLARS[a]

	Unfamiliarized (N)		Young familiarized (N)		Old familiarized (N)	
Consumed	CL	CD	CL	CD	CL	CD
Totally	27	49	18	24	42	32
Partially	8	0	18	10	6	7
Not	15	1	14	16	2	11
P	< 0.001*		0.19		0.02*	

[a] Data are compared by column using χ^2. *$\alpha < 0.05$. For definition of CL and CD, see Table 1.

in the hostplant (Table 1). MCSO and PCSO were found in lower quantities in the phytophagous insect than in the green leek leaves, with the exception of the exuviae.

Preliminary Behavioral Experiment. The preliminary experiment concerning choice between male and female caterpillars by unfamiliarized workers showed no significant difference between the two sexes. The numbers of males and females consumed in 24 hr were not statistically different (28% vs. 26% not consumed, 22% vs. 34% partially consumed, and 50% vs. 40% totally consumed; $\chi^2 = 2.229$, $P = 0.32$). It was, thus, possible to use sexual dimorphism as a marker of the origin of *A. assectella* caterpillars.

Behavior of Workers. When the unfamiliarized ants had the choice between a CL and a CD, the number of CD totally or partially consumed was greater than that of the CL (Table 2). After early dietary familiarization with CL, the young (1-month-old) workers showed no preference for either caterpillar category (Table 2). However, in 6- to 8-month-old workers, dietary familiarization with CL led to a tendency to prefer the CL (Table 2).

DISCUSSION

The concentrations of MCSO and PCSO in the caterpillars of the leek moth were generally lower than in the plant and showed a high variability, as indicated by the minimal and maximal values. This variability could come from the hostplant itself, as, the *Allium* genus displays large variations in the concentrations of these compounds, depending on the season, the variety, the organ, or the amount of sulfur in the soil, (Lancaster et al., 1984). While the concentration of these amino acids in the phytophagous insect depends on the plant, it is not an exact reflection of this. The caterpillar contains more PCSO than MCSO, although both are particularly abundant in the cuticle since the exuviae are rich in them, whereas the chrysalids contain little. This agrees with the results obtained in

certain Lepidoptera specialized on plants producing alkaloids in which the cuticle contains the majority of the alkaloids present in the caterpillars (Montllor et al., 1991). Moreover, a high quantity of the sulfur compounds found in the caterpillar must come via the digestive tract. The reduction in these compounds in starved caterpillars in comparison to those that have fed, probably comes from the presence of leek particles in the digestive tract. The accumulation of sulfur amino acids in the caterpillars of the leek moth makes plausible the previous hypothesis on the role of alkyl-cysteine sulfoxides of *Allium* in the protection of *A. assectella* against *Formica* ants (Nowbahari and Thibout, 1992). This role is confirmed in our choice tests, as the unfamiliarized *M. rubra* workers preferred the CD that had never been in contact with the leek.

After repeated encounters with the CL avoided prey, *M. rubra* workers chose their prey differently from unfamiliarized workers. They equally attacked the two categories of caterpillar and, in the case of the old workers, preferred the previously avoided caterpillars to those with which they had been familiarized, i.e., the CL. This adaptation phenomenon is not limited to any particularly sensitive period in young adults, as it is also found in old workers. Moreover it cannot be an imprinting process as has been observed several times in ants (Jaisson, 1975, 1980; Beckers et al., 1994; Schumann and Buschinger, 1994). It is more of a habituation process, which gradually leads to the disappearance and possibly to the inversion of the initial response of the unfamiliarized workers.

Based on the results obtained here, it would be interesting to study the behavior of the ants when they came into contact with the cuticle or the gut of the caterpillars that are rich in sulfur compounds.

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TASTE THRESHOLDS AND SUPRATHRESHOLD RESPONSES TO TANNIN-RICH PLANT EXTRACTS AND QUININE IN A PRIMATE SPECIES (*Microcebus murinus*)

S. IACONELLI and B. SIMMEN*

CNRS/FRE 2323, Muséum National d'Histoire Naturelle
Laboratoire d'Ecologie Générale
4 av. du Petit Château, 91800, Brunoy, France

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Abstract—Theories of plant chemical defenses discriminate between quantitative digestibility reducers (e.g., tannins) and qualitative toxins (e.g., alkaloids). Since the differential effect on taste of these compounds is poorly known, we recorded ingestive responses of a primate species, *Microcebus murinus*, to four tannin-rich plant extracts and to quinine, by using the behavioral method of the "two-bottle test." The efficiency of tannic extracts at precipitating protein was measured with the blue BSA method. Inhibition taste thresholds for tannins added to a moderately sweet solution varied between 0.25 and 2 g/l. The threshold for quinine hydrochloride was 0.32 g/l. The profiles of the response/concentration curves established for these astringent and bitter substances were similar, with maximal inhibition of consumption occurring for near-threshold concentrations. The large amounts of quinine required to deter this small-bodied species from feeding were unexpected, given its unspecialized frugivorous/insectivorous diet. We propose that the taste responses of *Microcebus* to tannins have been shaped in relation to the widespread occurrence of efficient polyphenols in food plants, while low responsiveness to quinine reflects a low risk of ingesting toxic alkaloids when feeding on ripe fruits and insects.

Key Words—Tannins, alkaloids, primate, taste, feeding deterrent, diet flexibility.

INTRODUCTION

Plant defense theories hold that plants evolved allelochemicals to protect themselves against consumers (Fraenkel, 1959), with different compounds adapted to different types of herbivores (Feeny, 1976) or were determined by endogenous

* To whom correspondence should be addressed.

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factors and nutrient availability (Coley et al., 1985). Although comparative studies in the field of chemical ecology suggest that primate species vary in their tolerance of tannins (e.g., Ganzhorn, 1988; Barton et al., 1993; Wrangham et al., 1998), the efficiency of polyphenols as deterrent substances to primates, remains debated (e.g., Maisels et al., 1994).

There are currently few experimental studies that have assessed whether tannins are rejected, tolerated, or even preferred by primates. To date, tannic acid, a hydrolyzable tannin, has been used as the prototypical stimulus of the astringent taste quality in electrophysiological and behavioral experiments. This polyphenol has a depressing effect on food intake of primates (Simmen, 1994; Simmen et al., 1999b; Iaconelli and Simmen, 1999; Laska, 2000). In contrast, reponses to bitter substances have been determined in many species, and reflect a wide distribution of sensitivities toward these distasteful compounds (for a reviews, see Glaser, 1986; Simmen and Hladik, 1998). Referring to the theories of plant chemical protection, one may wonder whether tannins, which are considered quantitative chemical defenses given their dose-dependent inhibitory activity on protein digestibility, and alkaloids, which are efficient at low concentration, elicit different tastes and are perceived at distinct gustatory thresholds.

The very existence of a taste quality elicited by tannins has been, and still is, disputed by many authors who consider the tactile dimension as the main sensory effect, namely the sensation of dryness in the mouth involving the precipitation of salivary proteins and stimulation of the mechanoreceptors of the tongue (McManus et al., 1981). However, the recording of pulses of the chorda tympani (one peripheral taste nerve), with a very short latency, in response to various tannins—while no responses could be observed in the lingual nerve—strongly suggested that the perception of these substances is multimodal and includes a taste component (Schiffman et al., 1992). In primates, the recording of chorda tympani whole nerve responses (*Microcebus murinus*) and of single fiber responses (*Callithrix jachus*) confirmed neuronal gustatory activity in the presence of tannic acid and indicated that the taste fibers responding preferentially to quinine and other substances tasting bitter to human were also those that responded best to the tannic substances (Hellekant et al., 1993b; Danilova et al., 1998).

In this study, we tested whether the taste threshold and suprathreshold responses obtained previously with tannic acid in a frugivorous/insectivorous species, the lesser mouse lemur *Microcebus murinus* (Simmen et al., 1999b), could be similar in other hydrolyzable tannins and condensed tannins, and, more generally, whether or not ingestive responses differed between polyphenols and alkaloids. The behavioral method of the "two-bottle test" was used, as it proved to yield thresholds comparable to those determined from electrophysiological recordings of the activity of the chorda tympani of *Microcebus murinus* (Simmen and Hladik, 1988; Hellekant et al., 1993a). Bearing in mind the likely interaction between tactile and taste sensations when recording ingestive responses to tannic solutions, we

use the term "astringent taste" as a descriptor of the global oral perception elicited by tannins.

METHODS AND MATERIALS

Rearing Conditions. Behavioral and physiological activities of *Microcebus murinus* are synchronized by daylength variations. In captivity, the breeding season occurs during the phase of long daylength (>12 hr), during which animals are very active, whereas complete gonadal regression, fattening, reduced behavioral activities, and lethargic state occur during the phase of short daylength (<12 hr). To ensure highly synchronized changes in biological activities among individuals over a complete cycle (Perret and Schilling, 1987), animals are routinely exposed to an artificial light cycle consisting of a 3-month period of short days (10L:14D) and a 5-month period of long days (14L:10D).

Experiments started 4 wk following the change between short daylength and long daylength, and they extended over the phase of long daylength. Subjects were sexually mature *Microcebus murinus* males, housed in separate cages (160 cm long × 75 cm wide × 250 cm high) and maintained under constant temperature (25°C) and relative humidity (55%). To reduce the time of isolation of individuals and to avoid impeding the breeding program necessary to the turnover of this primate colony, two distinct test groups were used in experiments. One group consisted of 14 animals tested with tannins, and another group included 9 animals tested with quinine.

Test Procedure and Statistical Analysis. The two-bottle test was used to determine the taste thresholds and suprathreshold responses to four tannin-rich plant extracts and one alkaloid in successive series of tests. The test procedure, derived from that of Glaser (1986), has been described in Simmen et al. (1999b) and is as follows: animals were provided with two feeding bottles, one containing 100 mM fructose solution, and the other containing the same sugar solution mixed with the deterrent substance. The concentration of the deterrent substance was varied at random in each test. Solutions were left at the beginning of the night phase, and the amounts of liquid consumed were recorded 1 hr later. The position of the bottles was determined at random to avoid side-preference effects. Tests were performed before animals were fed their daily meal of fruit, insects, cereal, milk, and fresh water. Before starting experiments with tannins and quinine, animals were provided simultaneously with tap water and a sugar solution at 100, 200, or 300 mM. Habituation to the two-bottle test was considered achieved when a marked preference for the sweet solution was recorded over four successive trials.

The threshold was defined as the lowest concentration of tannin or alkaloid for which the mean difference of consumption between the mixture and the pure fructose solution was significant ($P < 0.05$ with a paired-sample t test). Fructose was used rather than tap water because the volumes of water ingested by an

animal weighing less than 100 g are not reliable measures during short tests. The concentration of the fructose solution at 100 mM was chosen according to the response–concentration curve previously determined for this species: this concentration corresponds to twice the threshold for this sugar and exerts a slight attraction to animals (Simmen et al., 1999b).

The mean percentages of inhibition of the consumption of the mixtures were calculated and plotted against suprathreshold concentrations of tannins and quinine. According to previous investigations of the ingestive responses of *Microcebus* (and another mammal species) to tannic acid mixed with fructose (Simmen et al., 1999b; Iaconelli and Simmen, 1999), the use of a curvilinear regression model (Monod's model) proved to fit the patterns of inhibition with good consistency. Hyperbolic profiles were also obtained in the present study, and we applied this model to compare parameters of rejection among tannins and quinine. The theoretical equation is $y = (ax)/(b + x)$, in which a is the height of the asymptote tending toward 100% inhibition, and b is the concentration corresponding to $a/2$. The use of this model requires a translation of the data along the x-axis to comply with the condition $y = 0$ when $x = 0$. Indeed, the lack of inhibition ($y = 0$) is invariably observed for non-null concentrations. The highest concentration for which there is no inhibitory effect (that is, just below the statistical threshold) was subtracted from each concentration, and negative values were excluded before running the STATITCF program.

Compounds Tested. The fructose concentration at 100 mM (Sigma) in the binary solution and in the alternative feeding bottle (both 10 ml) yielded a caloric value of 0.675 kcal.

The substances tested were quinine hydrochloride (Prolabo) and tannin-rich extracts (powders) from oak bark, chestnut bark, and gallnut (Oenofrance). Together with these hydrolyzable tannins, crude quebracho tannin (provided by A. E. Hagerman) was also tested as a representative of condensed tannins. Different ranges of concentrations were used according to the compound tested: between 0.5 and 10 g/l for oak tannin, gallnut tannin, and quebracho tannin, between 0.05 and 10 g/l for chestnut tannin, and between 0.04 and 1.59 g/l (or 0.1 and 4 mM) for quinine hydrochloride.

Purified tannins other than tannic acid are not commercially available. The tannin powders tested clearly elicit an astringent taste and are used by food companies as taste exhausters for human foods and beverages. In our samples, the degree of purity was 75% in oak tannin, 81% in chestnut and quebracho tannins, and 96% in gallnut tannin. Since a rigorous analysis of taste perception of tannins must await further standardization of purification methods, we performed the blue BSA (bovine serum albumin) method of Asquith and Butler (1985) to compare the efficiency of extracts at precipitating protein. Solutions of 0.5 and 0.25 mg/ml of powders dissolved in diluted methanol served as substrates for precipitating 4 mg of BSA (Sigma; ref. A6003).

TABLE 1. MONOD'S PARAMETERS DESCRIBING INHIBITION OF CONSUMPTION OF MIXTURES AS A FUNCTION OF INCREASING CONCENTRATIONS OF DETERRENT SUBSTANCES

Mixture including	x' (g/l)	a (%)	95% confidence limits of a	b (g/l)	95% confidence limits of b	r^2
Chestnut tannin	0.125	82.3	[75–90]	0.18	[0.13–0.24]	0.99
Gallnut tannin	1.0	88.9	[71–100]	1.22	[0.91–1.53]	0.99
Oak tannin	1.0	82.5	[77–87]	1.22	[0.96–1.39]	0.99
Quebracho tannin	1.5	78.8	[71–86]	1.58	[1.43–1.73]	0.99
Quinine hydrochloride	0.278	81.7	[70–100]	0.279	[0.266–0.302]	0.99

[a] x': observed highest concentration for which no inhibition is observed; a: maximal inhibition; b: concentration of the deterrent substance corresponding to $a/2$, adjusted to x' (see Methods and Materials).

RESULTS

Tannins and quinine were clearly aversive to lesser mouse lemurs. As shown in Figure 1, the inhibition threshold was 0.25 g/l for chestnut tannin ($t = 3.99$, $N = 14$, $P < 0.001$), 1.5 g/l for gallnut tannin ($t = 3.72$, $N = 14$, $P < 0.001$), and 2 g/l for oak tannin and quebracho tannin (respectively, $t = 3.09$, $N = 14$, $P < 0.001$, and $t = 3.99$, $N = 14$, $P < 0.001$). The taste threshold for quinine hydrochloride was 0.32 g/l (or 0.80 mM; $t = 3.97$, $N = 9$, $P < 0.05$). The relative efficiencies of tannin extracts at precipitating bovine serum albumin were as follows: chestnut (1.82 mg/mg, on average, of BSA precipitated) > gallnut (1.52 mg/mg) > quebracho # oak (1.20 and 1.27 mg/mg). The thresholds for tannins tended to correlate negatively with protein precipitation efficiency, i.e., the stronger the precipitation properties, the lower the threshold (i. e. the higher the sensitivity; $r^2 = 0.946$; $P < 0.03$; $N = 4$).

The profiles of suprathreshold responses to increasing concentrations of quinine and tannins are expressed in Figure 2. The various compounds elicited similar profiles of inhibition of the ingestion of mixtures. The parameters of Monod's equation describing these profiles are summarized in Table 1. The correlation coefficients of the nonlinear regressions were high ($P < 0.01$), validating the use of an hyperbolic model. Maximal inhibition (a) was about 80% in all compounds—total inhibition is never observed because the choice procedure requires that animals lick both solutions. The concentration b, corresponding to $a/2$, varied between compounds and mirrored differences between thresholds, the lowest being for chestnut tannin and quinine hydrochloride.

The inhibition potency of the substances can be assessed by examining, in each case, to what extent b deviates from the threshold (relative index of tolerance). By definition, threshold concentrations determined statistically lay on a difference of consumption that, in our case, exceeded 50% (i.e., above $a/2$). Accordingly,

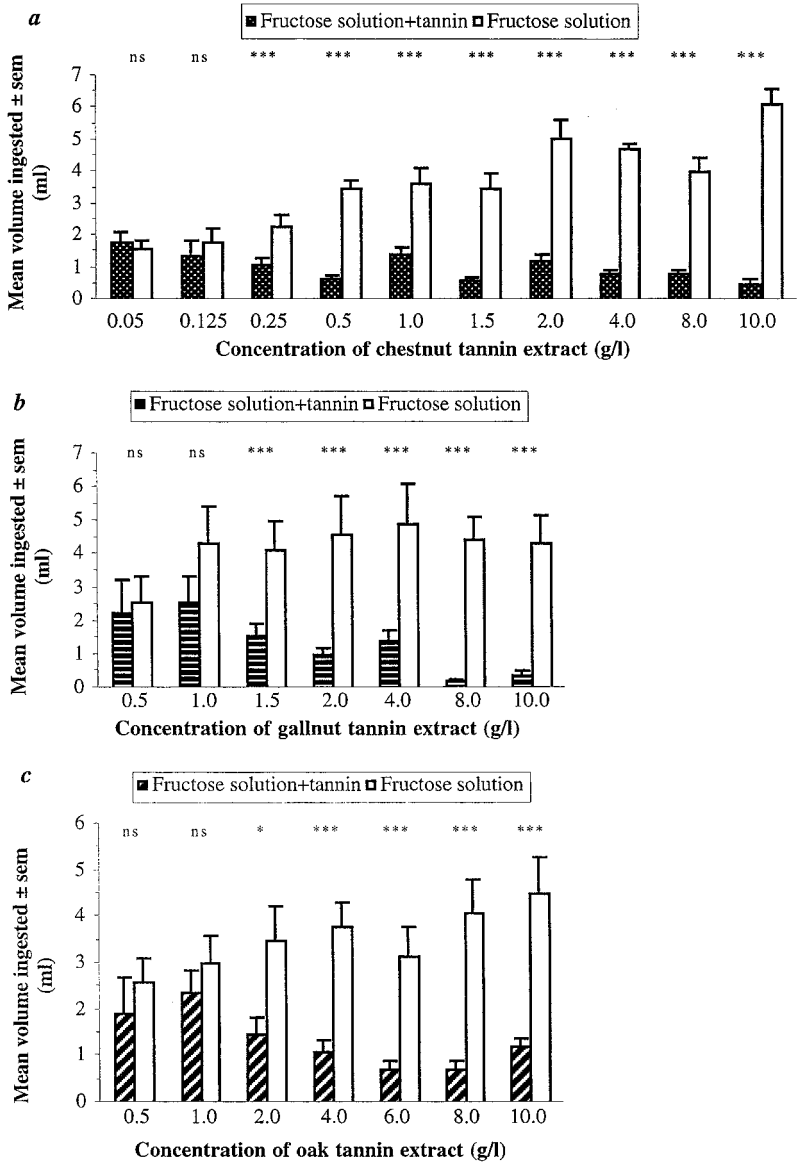


FIG. 1. Taste thresholds for tannin extracts and quinine. *a*: chestnut tannin, *b*: gallnut tannin, *c*: oak tannin, *d*: quebracho tannin, *e*: quinine hydrochloride. The threshold is defined as the lowest tannin or quinine concentration mixed with a fructose solution (100 mM) for which the mean difference of consumption between the mixture and the pure fructose solution is significant ($P < 0.05$ or lower in a paired-sample t test). The number of individuals tested at each concentration is 14 with tannins and 9 with quinine. ns: not significant, $^{*}P < 0.05$, $^{***}P < 0.001$.

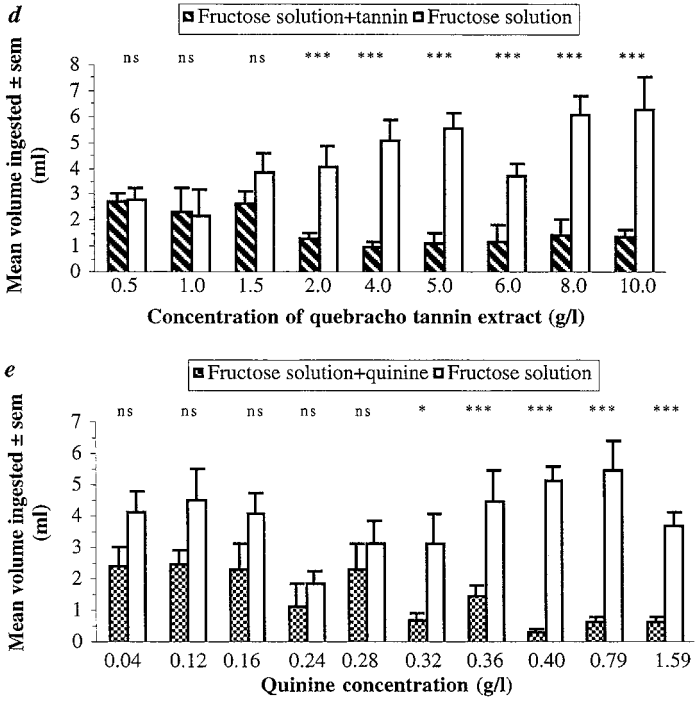


FIG. 1. Continued.

the ratio b/x', where x' is the concentration immediately below the statistical threshold, was calculated instead. This index was close to 1 for every compound tested (1.22:1 for oak and gallnut tannin; 0.18:0.125 for chestnut tannin; 1.58:1.5 for quebracho tannin; 0.279:0.278 for quinine hydrochloride), reflecting a strong rejection of the mixtures for near-threshold concentrations.

DISCUSSION

In a previous experiment on *Microcebus murinus*, we showed that the taste threshold for tannic acid mixed with sugar during the phase of long daylength (0.56 g/L) was close to the "electrophysiological" threshold (0.36 g/L) determined from responses of the chorda tympani proper nerve to pure solutions of tannic acid (Hellekant et al., 1993b; Iaconelli and Simmen, 1999). One may expect that threshold values presented here for other astringent substances (between 0.25 and 2 g/L) and determined during the phase of long daylength, probably closely reflect the minimal peripheral taste sensitivity toward these compounds.

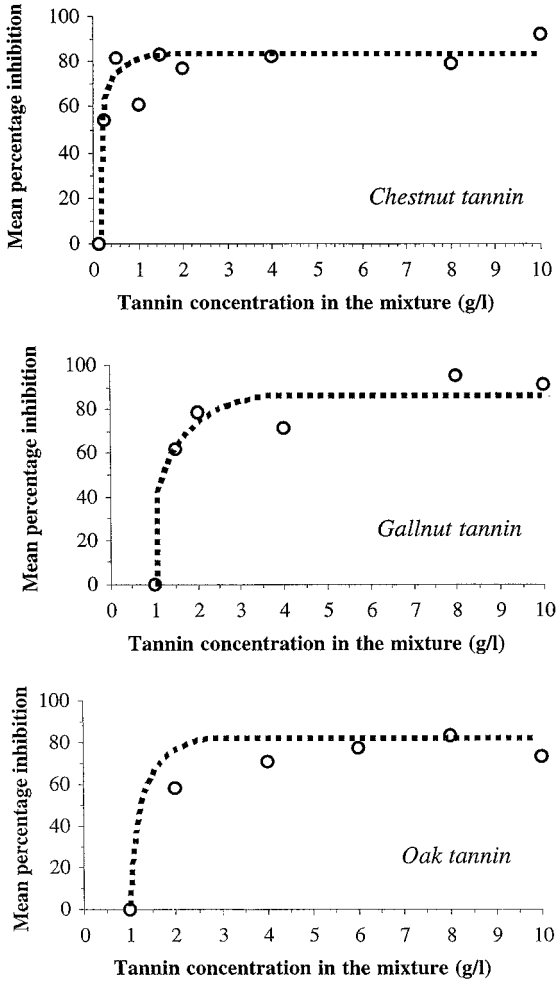


FIG. 2. Profiles of inhibition of the consumption of mixtures as a function of tannin and quinine concentration. Observed inhibition values are figured while curves are derived from parameters of Monod's model expressed in Table 1. As an example, no inhibition is observed for gallnut concentrations $\leq$1g/l, and the equation is $Y = a \cdot (X - 1)/(b - 1) + (X - 1)$, with $a = 88.9$ and $b = 1.22$. The resulting profile is an adjusted plot of calculated Y values against untransformed concentration values.

In other nonhuman primate species tested ($N = 6$) with a methodology identical to that used in this study (mixtures including a near-threshold sugar concentration), thresholds for tannic acid varied between 0.17 and 0.85 g/l (Simmen, 1994; Laska, 2000), with the exception of gorillas (6.8 g/l; (Remis and Kerr, 2002). In

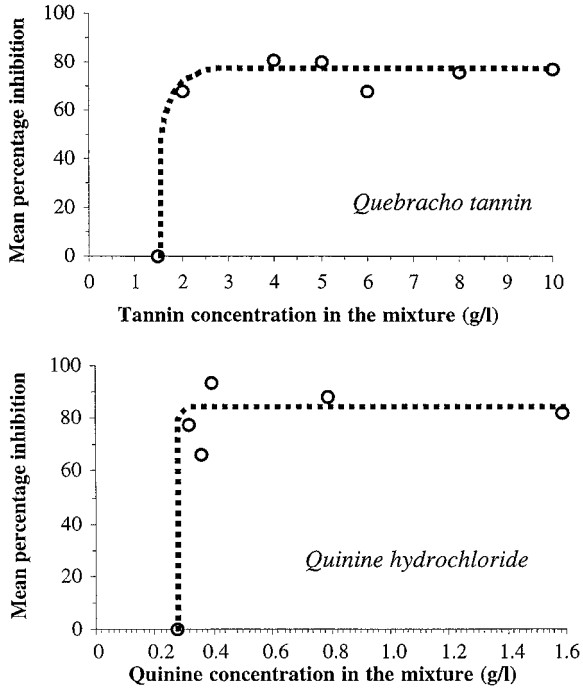


FIG. 2. Continued.

humans, recognition thresholds for tannic acid and oak tannin (from the same batch as the one we used) dissolved in tap water were found to be 0.34 g/l and 0.44 g/l, respectively (Iaconelli, 2000; Hladik et al., 2002). The clustering of thresholds for tannic acid found in most species tested to date contrasts sharply with the sensitivity to the bitter-tasting quinine. Using the two-bottle test, Glaser (1986) reported a threshold for quinine hydrochloride in aqueous solution at 0.32 g/l in *Microcebus*, a result that is strikingly equivalent to ours. This value is one of the highest found among primates, whose thresholds vary by a factor of 1–1000 (Simmen and Hladik, 1998). In addition, the profile of suprathreshold responses to quinine does not differ from those obtained with tannins, and approximately similar amounts of quinine and chestnut tannin (or tannic acid; see Iaconelli and Simmen, 1999) are required to fully deter *Microcebus* from drinking solutions.

Theories of chemical defenses (e.g., Feeny, 1976) separate, among allelochemicals, those whose deterrent effect is dose-dependent, such as polyphenols, from those that are biologically active at low concentration, such as alkaloids. Data obtained in *Microcebus* appear puzzling in this regard. Could it mean that Malagasy forests are poor in plants containing alkaloids, thus exerting little

selective pressure on the discrimination of bitter substances? Potent alkaloids have been extracted from Malagasy plants, and screening tests show that environments inhabited by *Microcebus murinus* and congeneric species include significant proportions of plants containing alkaloids in leaves (Ganzhorn, 1988; Simmen et al., 1999a). While these phytochemical studies indicate that the risk of ingesting poisonous substances is real for leaf-eating animals, *Microcebus murinus* may not be exposed to similar chemical constraints. This species includes predominantly ripe fruit pulps, nectars, gums, and invertebrates in its diet (Martin, 1972; Hladik et al., 1980). The lack of noxious substances in ripe fruits is predicted by theories of mutualism between plants and their dispersers (e.g., Herrera, 1985), and one may hypothesize that a limited risk of encountering harmful substances may stem indirectly from the primary attraction exerted by food items eliciting a sensory reward (including sweet and fatty fruits; Atsalis, 1999). It is significant that the congeneric species *M. rufus*, inhabiting the eastern rain forest of Madagascar, eat fruits lacking alkaloids and containing weak concentrations of condensed tannins (Ganzhorn, 1988). A high threshold can nevertheless contribute to the avoidance of potentially noxious substances. For instance, seeds are known to contain the highest concentrations of alkaloids among plant tissues (McKey, 1974), and inadvertant ingestion of smooth toxic seeds together with pulp is potentially deleterious (Simmen and Sabatier, 1996).

Polyphenols, unlike alkaloids, are widespread substances occurring in unripe fruits, barks, and gums among other plant organs (Swain, 1979). One may expect lesser mouse lemurs to cope more frequently with these allelochemicals than with alkaloids when monitoring fruit ripeness or when collecting gums oozing from tree barks. In this respect, the inhibition thresholds of *Microcebus* for tannins probably correspond to an efficient detection of these chemical defenses.

One aspect that must be mentioned is that the differences we found among taste thresholds for the four tannin extracts correlated negatively with differences in the efficiency of tannins at precipitating protein. This relationship, however, is not confirmed by data on tannic acid, for which a high sensitivity (Iaconelli and Simmen, 1999) corresponds to a weaker precipitation ability (1.04 mg of BSA under experimental conditions described for tannin extracts). It may be that, in the wild, digestibility-reducing effects of tannins are commonly predictable from the intensities of the astringent taste perceived, a relationship that would have been encoded in the gustatory system (see the gustofacial reflex triggered by quinine in primates) (Steiner and Glaser, 1984). In this case, some less toxic tannins eliciting a strong taste would also be rejected.

Despite the steepness of the responses to near-threshold tannin concentrations, *Microcebus murinus* is, to some extent, able to tolerate tannins according to the alimentary context. For instance, tannin–sugar mixtures that are rejected when given the choice between mixtures and a pure sugar solution, may be prefered within a range of above-threshold tannin concentrations (up to at least four fold

the threshold value) if water is presented simultaneously (Simmen et al., 1999b). Sensory tolerance may account for some observations of *Microcebus* feeding on tannin-rich fruits in Madagascar (Atsalis, 1999).

Our experiments provide some basis to assess the limits of diet flexibility. Considering the different profile of suprathreshold responses to tannic mixtures obtained in one other frugivorous–insectivorous mammal species (Simmen et al., 1999b), tolerance of distasteful compounds needs to be investigated in primate species showing distinct dietary trends.

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PREDICTING TOXICITY OF TALL LARKSPUR (*Delphinium barbeyi*): MEASUREMENT OF THE VARIATION IN ALKALOID CONCENTRATION AMONG PLANTS AND AMONG YEARS

M. H. RALPHS,[1,*] D. R. GARDNER,[1] D. L. TURNER,[2] J. A. PFISTER, and E. THACKER[1]

[1] *USDA/ARS Poisonous Plant Lab*
1150 E. 1400 North
Logan, Utah 84341, USA

[2] *USDA/Forest Service Rocky Mountain Research Station*
860 N. 1200 East
Logan, Utah 84321, USA

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Abstract—Tall larkspur (*Delphinium barbeyi*) is the principal mountain larkspur responsible for the majority of cattle deaths on mountain rangelands in western Colorado and central and southern Utah in the United States. Ten plants in each of two tall larkspur populations in the mountains near Ferron and Salina, Utah, were marked, and single stalks were harvested periodically through the growing season for 4 yr. Toxic alkaloid concentration [alkaloids containing the *N*-(methylsuccimimido)-anthranilik ester group] was determined by Fourier transform infrared (FTIR) spectroscopy. Individual larkspur plants varied in alkaloid concentrations, especially in early growth (14–38 mg/g). As the concentration declined over the growing season, variation among plants also declined. There were yearly differences in alkaloid concentration among individual plants ($P < 0.01$) and populations ($P < 0.001$), even after accounting for differences in phenological growth between years. Variables such as precipitation, temperature, days since snow melt, growing degree days (sum of mean temperature each day from snow melt), and plant height and weight were all considered in a Mallows Cp multiple regression selection procedure to predict alkaloid concentration. The mixed model procedure in SAS adjusted the regression equation for locations and years. Growing degree days was the best single predictor of alkaloid levels: $ln\ y = (3.581 - 0.00423\ \mathrm{GDD})$, $R^2 = 0.85$. Internal validation of this equation within individual years and locations from which the equation was developed, produced correlations between observed versus predicted values

* To whom correspondence should be addressed. E-mail: mralphs@cc.usu.edu

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ranging from $r = 0.73$ to 0.93. External validations on nine other larkspur populations produced correlations ranging from $r = 0.76$ to 0.99. This predictive equation can provide a tool for ranchers and land managers to make management decisions of when to graze cattle in larkspur areas.

Key Words—Tall larkspur, *Delphinium barbeyi*, *N*-(methylsuccinimido)-anthranilic ester, methyllycaconitine, poisonous plant, cattle.

INTRODUCTION

Tall larkspur (*Delphinium barbeyi*) is the principal mountain larkspur responsible for the majority of cattle deaths in western Colorado and central and southern Utah in the United States. Cattle deaths average 2–5% on mountain rangelands, but may exceed 15% in areas where larkspurs are abundant (Pfister et al., 1999). Larkspur plants contain many norditerpenoid alkaloids. Alkaloids containing the *N*-(methylsuccinimido)–anthranilic ester group (MSAL) are most toxic, with methyllycaconitine (MLA) being the most prominent toxic alkaloid (Manners et al., 1995). MLA is a potent neuromuscular blocker, causing paralysis and rapid death from respiratory failure (Dobelis et al., 1999).

Concentrations of alkaloids decline in a concave curvilinear manner as the plants mature through the phenological growth stages over the growing season (Pfister et al., 1994; Ralphs et al., 1997). Concentrations vary year to year (Pfister et al., 1994; Ralphs et al., 1997). It is not known if this results from differences in alkaloid production or simply from differences in phenological growth stages when plants were harvested. Alkaloid levels also differ greatly among plants (Manners and Pfister, 1996).

The first objective of this study was to quantify the variation in toxic alkaloid concentration among individual plants, over the growing season, and among years, then to describe the variation in populations over years and between sites. The second objective was to evaluate various plant and environmental factors that influence growth and determine their ability to predict alkaloid concentration in tall larkspur.

METHODS AND MATERIALS

Tall larkspur plants were sampled at two locations in the mountains of central Utah. The Ferron Reservoir site was 46 km west of Ferron, Utah, at an elevation of 3150 m in the subalpine zone. The second site, Mt. Terrell, was 40 km east of Salina, Utah, at 3230 m, in the ecotone of the aspen and subalpine zone.

Ten uniform tall larkspur plants (30–40 stalks/plant) were randomly selected at each site in 1997 and marked with an orange tent stake with an aluminum number attached. One randomly selected stalk was harvested from each plant on

a weekly basis, beginning shortly after emergence in late June, until senescence in September. Stalks were taken from the same plants at various times during the growing seasons over a 4-yr period. The height and weight of each stalk was measured when harvested, then placed in an airtight plastic bag and immediately frozen with dry ice. Samples were freeze dried, weighed to obtain a dry weight, and ground through a Wiley mill with a 1-mm screen. Alkaloids were extracted, and the concentration of toxic (MSAL) and total alkaloids was measured by Fourier transform infrared (FTIR) spectroscopy (Gardner et al., 1997) This paper deals only with the toxic MSAL fraction.

It was important to determine when larkspur growth began each year in order to compare phenological plant development between years. However, access to these sites early in the season was prevented by snow drifts. Weather recording sites (SNOTEL, automated weather recording sites operated by the Natural Resource Conservation Service) were located 3 km from the Ferron site and 7 km from the Salina site. The pillow data parameter measured weight of the snow pack as an estimate of water content. When this parameter went to 0 in the spring, it signaled the snow pack was gone. Larkspur growth begins immediately after snow melt (Cronin, 1971). The distance of the SNOTEL sites to the research plots precludes precise estimates of the beginning of growth, but it is the best estimate available and a consistent parameter over years. Time of snow melt and subsequent beginning of growth ranged from 5 May to 21 June, across locations and years of the study. Therefore, time was normalized by expressing time as days since snow melt. Daily temperature and precipitation data were also taken from the weather stations.

Variation. To quantify the difference in alkaloid concentration among plants, population summary statistics were calculated (mean, standard error, coefficient of variation, maximum and minimum value, and range of toxic alkaloid concentration) and are presented in Table 1 for 10 plants collected at the same stage of growth each year (flower stage). In addition, toxic alkaloid concentrations of individual plants were graphed to illustrate the range of variation over the 1997 growing season for the two locations (Figure 1).

A single plant was selected from each location to express the variation within the same plant between years. A mixed model analysis of variance using first-order autocorrelation covariate structure (SAS, 2001) was used to compare years. The MSAL values were transformed to the natural log (*ln*), and regressions were calculated by including days since snow melt as a covariate in the model and asking for its solution. Differences in these regressions between years were determined by linear contrasts. The raw data were graphed to illustrate differences in these selected plants between years (Figure 2).

The difference in alkaloid concentrations of tall larkspur populations between locations and years was evaluated in a mixed model analysis of variance similar to that described above. The MSAL data were log transformed, and linear regressions were calculated and compared between sites and among years using

linear contrasts. These log regressions were graphed to illustrate differences in populations over years (Figure 3).

Prediction. The following plant parameters and weather data were considered in multiple regression predictive equations. Plant height was measured when the plants were harvested. Stalks were freeze dried, and dry weight was obtained before extraction of alkaloids. Temperature, precipitation, and time of snow melt were taken from SNOTEL sites. Time was normalized over the 4 yr by expressing it as days from snow melt. Temperatures were converted to heat units or thermal time (measured in growing degree-days since snow melt, GDD). GDD have been used to describe and predict various phenological events in plants (reviewed by McMaster and Wilhelm, 1997) and are calculated as:

$$\text{GDD} = (T_{\max} - T_{min})/2 - \text{base}$$

where $T_{\max}$ and T_{min} are maximum and minimum daily temperatures. Base 4.4/25°C (40/70°F) was selected to represent growing conditions for cool-season species (Ashcroft et al., 1992).

Several statistical methods were used to develop predictive equations. Mallows Cp multiple regression model selection procedure was used to select the variables for the predictive equation (SAS, 2001) using the raw data. This procedure selects for the smallest total squared error and equations with the highest R^2 were selected.

Since alkaloid concentration declined in an exponential curvilinear fashion, the natural log transformation was made of toxic MSAL alkaloid concentrations, and linear regressions were calculated for each parameter. The classification and regression tree (CART) program was used on the log transformed data to define the changes in alkaloid concentration. Finally, the mixed procedure in SAS (SAS, 2001) was used on the log-transformed data to adjust the linear regression equation for the location and year factors in the collection scheme.

Two validations were run on the predictive equations. An internal validation was run on the same data set from which the equation was derived for the two locations each year. Correlations between the observed and predicted values were used to determine the precision of the equations. The accuracy of the equations was evaluated by calculating the mean variance $[\sum (\text{observed - predicted})^2]/n$. The lowest mean variance was most accurate.

An external validation was run on nine other larkspur populations. One population at each of the Ferron and Salina locations was in phenological growth stage similar to the population from which the equations were developed in 2000. The other population at each location in 2000 began growth earlier or later than the equation population (due to when the snow melted). These two data sets were used to determine the effect of different growth rates by using the same recorded environmental variables. Five other data sets from previous grazing studies were

TABLE 1. VARIATION IN TOXIC ALKALOID CONCENTRATION (MG/G DRY WEIGHT) IN FLOWER STAGE AMONG LOCATIONS AND YEARS ($N = 10$)

		Concentration					
Location	Year	Mean	SE	Max	Min	Range	CV[a]
Ferron	1997	3.40[b]	0.17	4.10	2.30	1.80	16
	1998	4.72[a]	0.43	8.20	3.80	4.40	28
	1999	4.68[a]	0.31	6.19	3.43	2.76	21
	2000	3.04[b]	0.23	4.15	1.81	2.34	24
	Mean	3.96[B]					
Salina	1997	4.85[a]	0.40	7.70	3.40	4.30	26
	1998	5.61[a]	0.53	9.40	4.0	5.40	30
	1999	4.87[a]	0.58	6.82	1.64	5.18	36
	2000	3.93[a]	0.49	5.92	2.07	3.85	33
	Mean	4.82[A]					
Manti[c]	1993	4.5	0.25	4.90	3.2	1.7	40
Yampa[c]	1993	5.5	0.28	6.7	4.6	2.1	45

[a] Coefficient of variation.
[b] AB Difference between locations ($P = 0.04$); ab difference between years within locations ($P < 0.05$).
[c] Data from Manners and Pfister (1996).

available in which we had the concentration of the toxic alkaloid MLA to compare to predicted values. Correlations between observed and predicted values, and mean variances were used to measure goodness of fit.

RESULTS

Variation among Plants. The toxic alkaloid concentration in tall larkspur plants harvested at the same phenological growth stage (flower stage of growth) at two locations over 4 yr varied from 1.64 to 9.40 mg/g (Table 1). The range of alkaloid concentration was almost as great as the mean. The coefficient of variation (CV, % variation about the mean) of plants in our study harvested at the flower stage of growth each year ranged from 21% to 28% at Ferron and from 26% to 36% at Salina (Table 1). Manners and Pfister (1996) reported the CV of 100 tall larkspur plants sampled in the early flower stage at Manti, Utah (near our Ferron site) and Yampa, Colorado ranged from 40 to 45%.

The relative ranking of alkaloid concentration in individual plants in 1997 was similar over the growing season (Figure 1). Variation among plants containing the highest and lowest amounts of alkaloid was greatest in the early growth (Ferron: 34.4 vs. 17.3 mg/g; Salina: 38.4 vs. 14.6 mg/g, respectively). By about 50 days after snow melt, variation among plants had diminished (Figure 1). Plants also

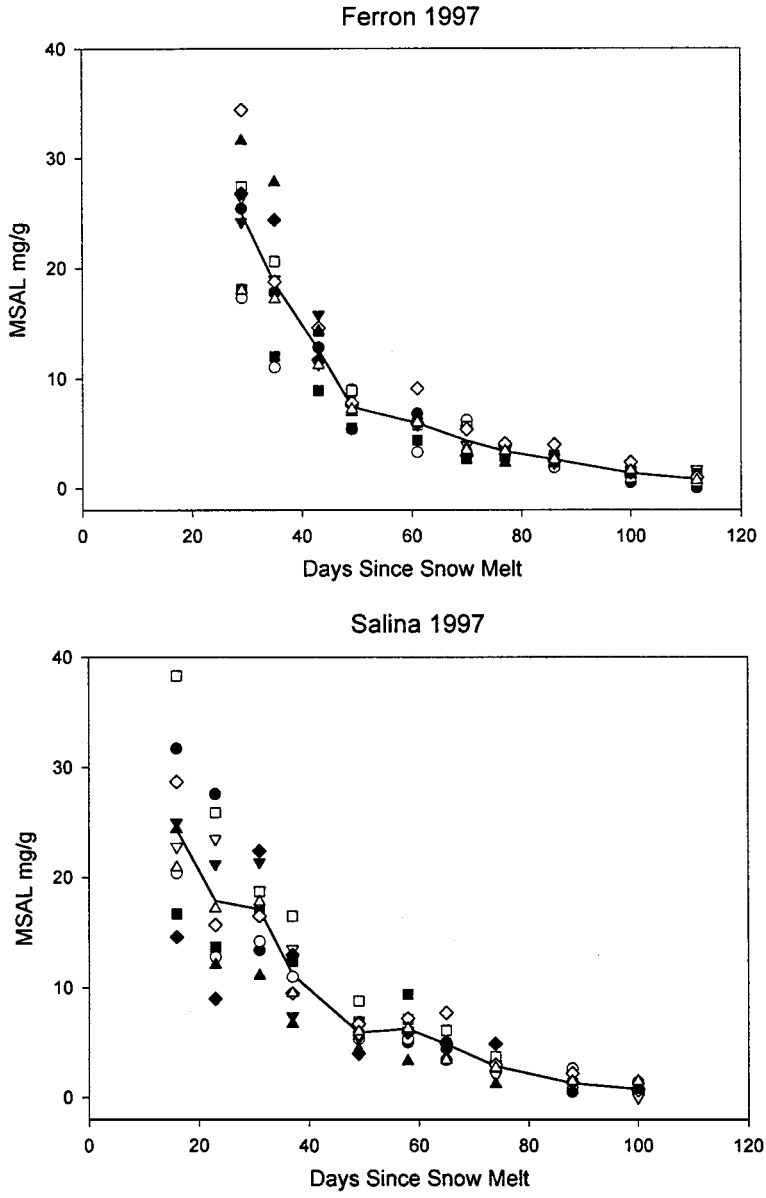


FIG. 1. Range in toxic MSAL alkaloid concentration in 1997 over the growing season at Ferron and Salina. Individual plants are denoted by different symbols ($N < 10$).

maintained their relative ranking in alkaloid concentration over years (data not shown). They varied slightly in numerical ranking, but generally those highest in toxic alkaloids had high levels all 4 yr.

Variation within the Same Plant over Years. Toxic Alkaloid concentration within the same plant varied over years. One plant from each location was selected to illustrate the variation (Figure 2). At Ferron, alkaloid levels of plant 198 differed in 1999 and 2000 ($P = 0.01$). At Salina, alkaloid concentration of plant 367 in 1997 differed from that in 1998 and 1999 ($P = 0.002$). Alkaloid levels at the beginning of the season in 1998 at both locations were somewhat of an anomaly. Levels were low during the first two collections, compared to 1997. June of 1998 was very cold and wet (mean temperature was 4°C lower than 1997 and 5°C lower than the 10-yr average, Ralphs et al., 2000). We speculate that alkaloid synthesis was suppressed during early growth in 1998. Collections in 1999 and 2000 began later in the season and do not reflect the high levels of early growth.

Population Variation between Locations and Years. Overall, there was a difference in alkaloid concentration between locations. Concentration over years and seasons was higher at Salina (8.0 mg/g) than at Ferron (6.9 mg/g) ($P < 0.001$, data not shown). There was also a difference in alkaloid concentration between years ($P < 0.001$). At Ferron, the concentration in 1999 was lower, and the slope of the log regression was less than in the other years (Figure 3). At Salina, concentrations were lower throughout the season in 1998 and 1999 than in the other 2 yr. This suggests that after adjusting for differences in phenology and time for growth, alkaloid concentrations differed in larkspur populations between locations and years.

Predictive Equations. The best single-factor equations selected by the Mallows Cp selection procedure to predict alkaloid concentration were plant height ($R^2 = 0.63$), days since snow melt ($R^2 = 0.68$), and growing degree days since snow melt ($R^2 = 0.72$). The best two-factor equation included growing degree days and plant height ($R^2 = 0.73$). The equations are listed in Table 2.

TABLE 2. PREDICTIVE REGRESSION EQUATIONS

Parameters	Equation	R^2
Raw data		
Plant height	$\hat{y}^a = 23.585 - 0.31\ \text{ht} + 0.00112\ \text{ht}^2$	0.63
Days since snow melt	$\hat{y} = 26.42 - 0.4626\ \text{day} + 0.0021\ \text{day}^2$	0.68
Growing degree days	$\hat{y} = 26.05 - 0.06\ \text{GDD} + 0.0000369\ \text{GDD}^2$	0.72
Height × growing degree day	$\hat{y} = 23.64 - 0.19\ \text{ht} + .000828\ \text{ht}^2 - 0.01568\ \text{GDD}$	0.73
Log transformed data		
Days since snow melt	$ln\ \hat{y} = 3.855 - .03732\ \text{day}$	0.79
Growing degree days	$ln\ \hat{y} = 3.581 - 0.00423\ \text{GDD}$	0.85
Back-transform data to natural scale	$\hat{y} = \exp(ln\ y)$	

[a] Predicted MSAL concentration.

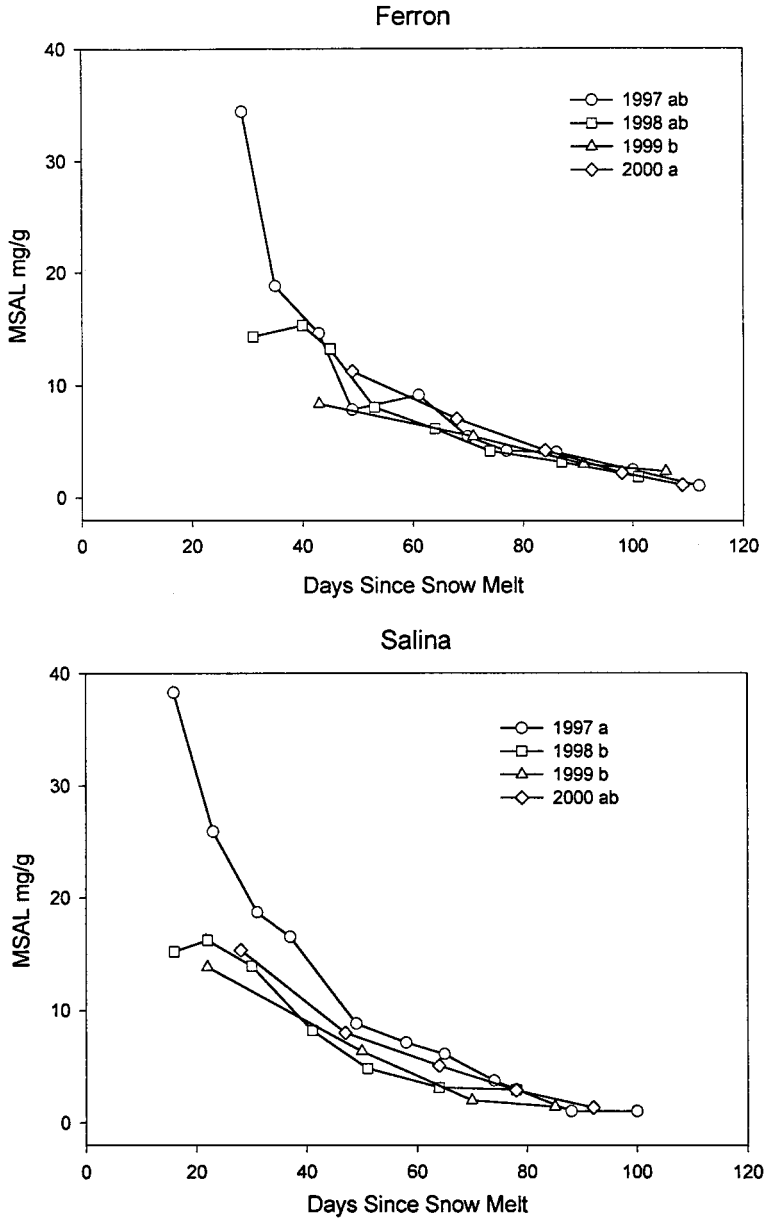


FIG. 2. Difference in toxic MSAL alkaloid concentration in the same plant between years at Ferron and Salina. Different letters following the year indicate significant difference ($P < 0.05$).

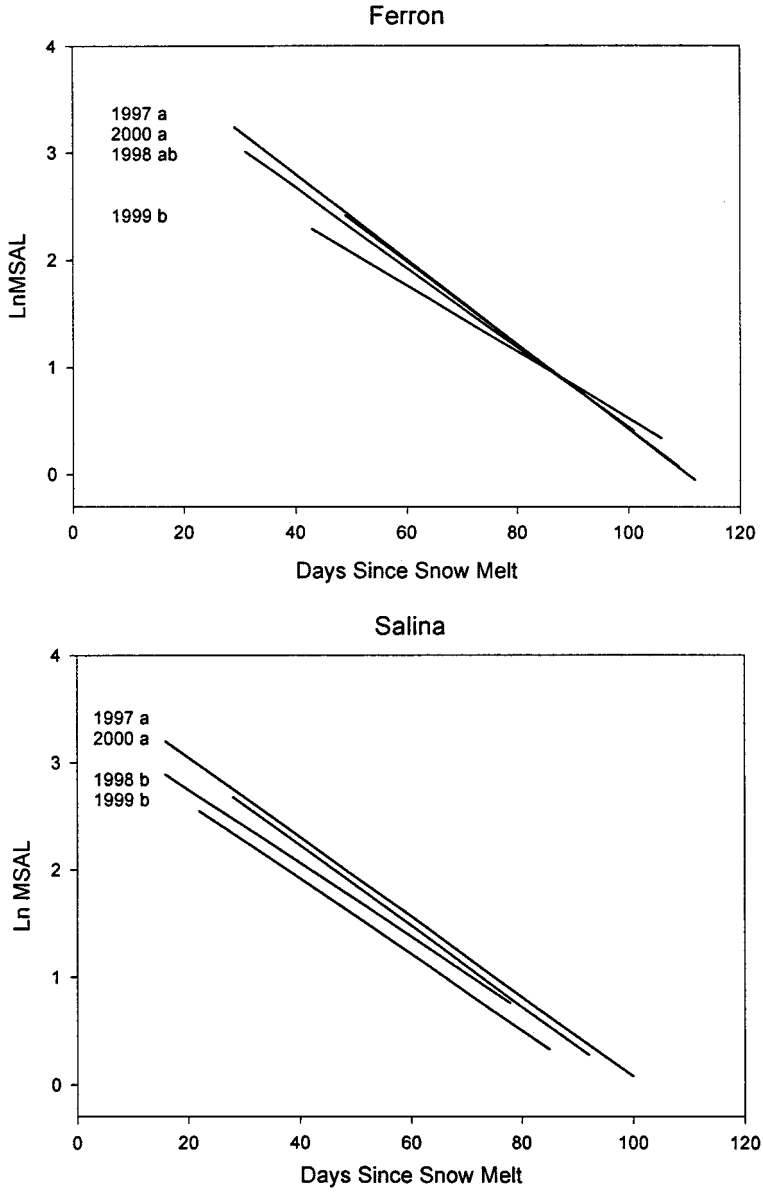


FIG. 3. Population difference between years, expressed as the natural log transformation of toxic MSAL alkaloid concentration for Ferron and Salina. Different letters following the year indicate significant difference ($P < 0.05$).

Growing degree days since snow melt (GDD) was the best single predictor in all of the other procedures. The CART analysis using GDD produced a 7-node tree with an R^2 of 0.84. The mixed procedure in SAS using GDD on log transformed data produced a regression equation with an estimated $R^2 = 0.85$ (Table 2). This was selected as the best and simplest prediction equation based on the highest R^2 value.

The predicted data generated by GDD in the SAS mixed procedure were back-transformed to the natural scale and compared with the observed data in the internal validation (Figure 4) and external validation data sets (Figure 5). Correlations between the observed and predicted values provided a measures of the precision of all the equations (Table 3), and the mean variances provide a measure of the accuracy of the equations (Table 4) and are presented for both the internal and external validation sets.

DISCUSSION

Growing degree days have been used extensively in agronomy and weed science to describe and predict phenological events (see McMaster and Wilhelm, 1977 for review). GDD were used to predict the time of flowering in low larkspur (*D. nelsonii*), and the number of flowers and seed production (Inouye and McGuire, 1991). Growing time (as expressed in Julian days since 1 January) was used to describe toxic alkaloid concentrations in the tall larkspur in Canada (*D. brownii)* in its vegetative stage (Majak et al., 2000):

$$ln\text{MLA} = 6.10059 - 0.031\text{Jday},\ R^2 = 0.79$$

The predictions of toxic alkaloid concentrations, using GDD since snow melt in the log-transformed SAS mixed equation in our study, were fairly close to the observed levels in most of the internal and external validation data sets. One anomaly occurred in the internal validation set in 1998 at both Ferron and Salina (Figure 4). Mean June temperatures were 5°C lower than average and may have suppressed metabolism during the first 3 wk of growth when alkaloids are synthesized (Ralphs et al., 2000).

Another anomaly occurred in the 2000 external validation set. The younger population at Ferron started growth later than the population near the weather station; thus, it was in an earlier phenological growth stage, and the observed toxic alkaloid levels should have been higher than the predicted levels (Figure 5). The older population at Salina was at a lower elevation and began growth earlier, thus, plants were more mature. Alkaloid concentrations were lower than predicted, as would be expected.

The predicted values of MLA in the older data sets at Manti in 1988, 1989, and 1991 were close to the observed values. The predicted value of MLA in the

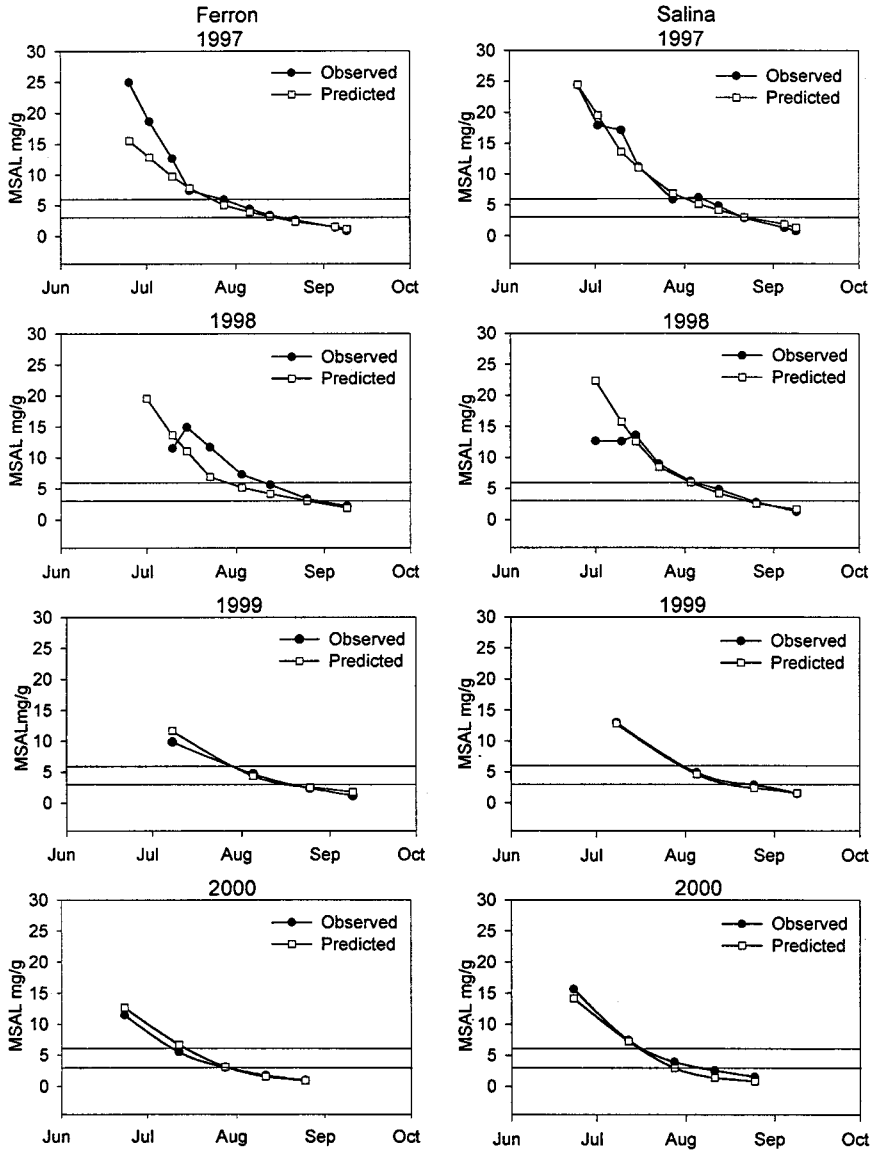


FIG. 4. Internal validation of log transformed predictive equation using growing degree days (GDD) since snow melt. Comparison of observed and predicted values from the same data set. Horizontal lines denote moderate and low toxic level thresholds.

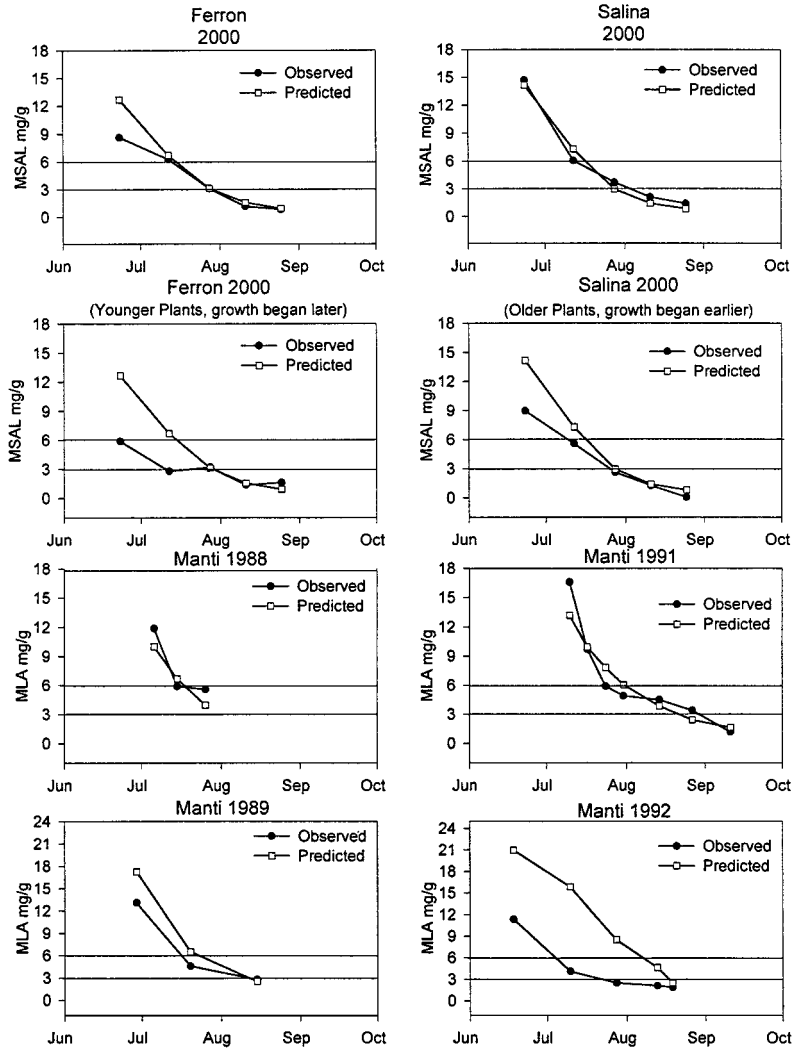


FIG. 5. External validation of the log transformed predictive equation using growing degree days (GDD) since snow melt of the 1997–2000 data set. Comparison of the observed values in previous years with the predicted values from the equation. Horizontal lines denote moderate and low toxic level thresholds.

1992 Manti and Salina data sets deviated from the observed. The correlations were close (Table 3), but the variances were an order of magnitude greater than in other years (Table 4). In this case, the predicted values appear to be invalid. These older studies measured only MLA, which is the major toxic alkaloid. The newer

TABLE 3. CORRELATION BETWEEN ACTUAL AND PREDICTED ALKALOID LEVELS IN MULTIPLE REGRESSION EQUATIONS USING RAW DATA, AND MIXED MODEL LOG SCALE REGRESSIONS

			Correlation coefficient (r)			
			Multiple regression		Mixed model regression	
Validation data set	Location	Year	Plant height	Height GDD[a]	Days since snow melt	GDD[a] growing degree days
Internal	Ferron	1997	0.93	0.93	0.93	0.93
		1998	0.73	0.80	0.77	0.76
		1999	0.81	0.93	0.93	0.93
		2000	0.47	0.77	0.84	0.84
	Salina	1997	0.84	0.90	0.90	0.90
		1998	0.64	0.75	0.97	0.73
		1999	0.86	0.93	0.93	0.93
		2000	0.88	0.90	0.91	0.91
External	Ferron	2000	0.72	0.77	0.79	0.78
		Young[b]	0.26	0.75	0.75	0.76
	Salina	2000	0.16	0.87	0.87	0.88
		Older[b]	0.87	0.87	0.89	0.89
	Manti	1988		0.88	0.90	0.91
		1989		0.98	0.98	0.99
		1991		0.92	0.94	0.94
		1992		0.82	0.98	0.87
	Salina	1992		0.91	0.97	0.95

[a] Growing degree days since snow melt.
[b] Population was phenologically younger or older than the population where weather measurements were taken.

chemical assays measure the entire MSAL toxic fraction, which may include low amounts of other alkaloids having similar toxicity.

Pfister et al. (1997) defined thresholds of toxicity in tall larkspur. Alkaloid concentrations <3 mg/g were considered low to nontoxic. Concentrations of 3–6 mg/g were considered moderately toxic and could cause poisoning if a cow consumed tall larkspur for 20–35% of her daily diet. Below the moderately toxic threshold, the SAS mixed model equation that used GDD predicted toxic alkaloid concentration accurately.

Online weather data is available from SNOTEL, or can be readily obtained from the states' NRCS water forecasting offices. By observing when snow melted from specific larkspur populations or using the snow melt data from SNOTEL and the maximum and minimum temperature, toxic alkaloid concentration can be estimated at any point in time. Conversely, the alkaloid concentration will drop below the moderately toxic threshold level (6 mg/g) when GDD since snow melt reach 422 (90% confidence interval 385–509), and the low toxicity threshold (3 mg/g) will be reached at GDD 586 (90% confidence interval 546–671).

TABLE 4. ACCURACY OF PREDICTIVE EQUATIONS[a]

			Variance			
			Multiple regression		Mixed model regression	
Validation data set	Location	Year	Plant height	Height × GDD	Days since snow melt	GDD[a]
Internal	Ferron	1997	14.45	**13.69**	19.0	19.48
		1998	9.76	**5.35**	13.5	5.98
		1999	4.51	2.50	**2.02**	2.30
		2000	18.40	9.14	9.96	**6.52**
	Salina	1997	21.97	15.23	**12.48**	12.59
		1998	13.28	**7.43**	46.34	8.36
		1999	6.68	3.36	19.41	**2.67**
		2000	8.37	6.50	**5.48**	6.22
External	Ferron	2000	8.86	13.97	**2.28**	13.55
		young	21.35	8.96	**7.35**	8.99
	Salina	2000	12.89	**3.82**	17.25	8.33
		older	7.74	7.24	7.17	**5.99**
	Manti	1988			5.62	**2.30**
		1989			19.87	**7.07**
		1991			2.54	**2.63**
		1992			**2.24**	54.73
	Salina	1992			**11.97**	59.59

[a] Variance Calculated as the Mean Difference of the Observed-Predicted Value. Lowest Variance (bold type) Indicates Best Fit. GDD: growing degree days since snow melt.

These generally correspond to the late flower and early pod stages of growth, respectively.

In Summary, individual larkspur plants varied in alkaloid concentrations, especially in early growth. In 1997 when plants were sampled shortly after they emerged from the snow (5–12 cm tall), alkaloid levels varied by over 100% (from 14 to 38 mg/g). Alkaloid levels declined in a curvilinear manner over the growing season. The high concentrations of alkaloids at the beginning of growth were diluted in the increasing plant biomass through the short growing season. As the alkaloid concentration declined over the season, variation among plants also declined. After accounting for differences in phenology and time for growth, both individual plants and populations varied among years. The predictive equation based on growing degree days from snow melt can accurately predict alkaloid levels through the critical regions as toxicity declines from moderate to low toxic thresholds. This predictive equation can provide a tool for ranchers and land managers to make management decisions of when it is safe to graze cattle in larkspur areas.

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METHYL JASMONATE AS AN ALLELOPATHIC AGENT: SAGEBRUSH INHIBITS GERMINATION OF A NEIGHBORING TOBACCO, *Nicotiana Attenuata*

CATHERINE A. PRESTON,[1,2,4] HAZEL BETTS,[3] and IAN T. BALDWIN[1,2*]

[1]*Max-Planck-Institute for Chemical Ecology, Beutenberg Campus*
Winzerlaer Strasse 10
07745 Jena, Germany

[2]*State University of New York at Buffalo*
Department of Biological Sciences
109 Cooke Hall/North Campus
Buffalo, New York 14260-1300, USA

[3]*University of Oxford*
Department of Plant Sciences
South Parks Road
Oxford OXI 3RB, England

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Abstract—*Artemisia tridentata* ssp. *tridentata* is the dominant and defining shrub in the Great Basin Desert, with well-documented allelopathic tendencies that have generally been ascribed to its most abundantly released secondary metabolites. However, as a minor component, sagebrush releases a highly biologically active substance, methyl jasmonate (MeJA), which is known to function as both a germination inhibitor and promoter in laboratory studies. *Nicotiana attenuata* is a tobacco species native to the Great Basin Desert and grows in newly burned juniper–sagebrush habitats for 2–3 yr following a fire. With a combination of field and laboratory studies, we examined the role of MeJA release from sagebrush by both air and water transport in inhibiting *N. attenuata* seed germination. We demonstrated that sagebrush interacts allelopathically with the seed bank of *N. attenuata* through its release of MeJA. In the field, seeds buried 0–40 cm from sagebrush plants for 4 months in net bags had significantly reduced germination compared to seeds buried similarly but protected in plastic bags. Moreover, germination on soils collected from underneath sagebrush plants was reduced by 60% compared to seeds placed on soils collected between sagebrush plants or outside of the sagebrush population. Exposure to *A. tridentata* seeds and seedlings did not affect *N. attenuata* germination, suggesting that

*To whom correspondence should be addressed. E-mail: baldwin@ice.mpg.de
[4]Present address: USDA-ARS CMAVE, 1600/1700 SW 23rd Drive, Gainesville, Florida 32608, USA.

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established sagebrush plants only influence the tobacco's seed bank. In the laboratory, exposure of seeds to sagebrush emissions resulted in germination delays of up to 6 d. Exposure to volatile and aqueous MeJA also inhibited germination of *N. attenuata* seeds at quantities that are released naturally by sagebrush: 3.5 μg/hr and 1.12 μg/seed cup (56 ng/seed), respectively. *A. tridentata* seeds were significantly more resistant to MeJA, being inhibited at 336 μg MeJA (16.8 μg/seed), 300 times greater than the level of aqueous MeJA required to inhibit *N. attenuata* seeds. MeJA inhibited *N. attenuata* germination regardless of the seed's dormancy state and the specific epimer (*trans*- or *cis*-) of MeJA. Germination on sagebrush chaff that had been heated to reduce MeJA content was negatively correlated with the amount of MeJA remaining in the chaff. Germination of a nondormant, conspecific tobacco, *N. trigonophylla*, which grows in the same area but is not associated with fire, is less sensitive than *N. attenuata* to the extracts of sagebrush litter, but similarly sensitive to MeJA. Additionally, four of five other tobacco species that are not known to be associated with sagebrush are less sensitive to MeJA, suggesting an evolved sensitivity to MeJA. To determine the proportion of germination inhibition of a sagebrush extract that could be attributed to MeJA, we serially diluted sagebrush extracts with water and restored the quantity of MeJA of the original extract by adding appropriate quantities of synthetic MeJA; 16–60% of the inhibitory activity of the original extract could be attributed to the MeJA. We conclude that MeJA release from sagebrush plays an allelopathic role for *N. attenuata* seed banks, but other unidentified compounds are also involved.

Key Words—*Artemisia tridentata* ssp. *tridentata*, *Nicotiana attenuata*, *Nicotiana trigonophylla*, allelopathy, methyl jasmonate, germination, seed dormancy.

INTRODUCTION

Allelopathy is defined as a direct or indirect interaction, whereby chemicals released by one organism influence the physiological processes of another neighboring organism (Molisch, 1937; Rice, 1984), and is one of the most controversial of ecological interactions (Harper, 1977; Williamson, 1990). Much of the controversy involves the requirements for proof of an allelopathic interaction and the difficulty of disentangling allelopathy from other ecological relationships, such as resource-based competition (Harper, 1977; Williamson, 1990; Inderjit and del Moral, 1997; Watkinson, 1998). Allelopathy and resource competition are likely to interact in complicated ways, as determined by the complicated effects of variable resource supply on secondary metabolite production and release (Rice, 1984; Bryant et al., 1983; Inderjit and del Moral, 1997). Hence, allelopathic interactions, however minor in themselves, may have large effects by changing the outcome of resource-based competition (Reigosa et al., 1999). Moreover, patterns of plant distribution in nature that have been ascribed to allelopathic interactions, could, after closer scrutiny, be attributed to other ecological interactions. For example, the bare zones around chaparral shrubs, initially thought to result from allelopathic interactions (Muller, 1965), could also be explained by the removal of seeds by

rodents (Bartholomew, 1970). These complications make field-based demonstrations of allelopathy particularly onerous.

Skepticism about the importance of allelopathic interactions in nature has focused attention on the methods employed to demonstrate them (Harper, 1977) and consequently, studies of allelopathy tend to be held to a more rigorous standard of proof than other plant–plant interactions (Harper, 1977; Williamson, 1990). A credible allelopathic interaction must first involve ecologically relevant species that interact in nature. Results from studies that use "standardized" bioassay species, such as lettuce or radish, are difficult to generalize to co-occurring species in natural populations (Inderjit, 1996). The fundamental bioassay technique in allelopathic research of using whole-plant extracts, foliar washings, and decomposing litter was questioned, as these treatments inhibited germination or growth even when derived from plants not suspected of allelopathy, and hence suggested antagonistic relationships between species that could not be associated with patterns found in nature (Stowe, 1979). Second, the chemical(s) that mediates the interaction must be identified and the amounts released into the environment quantified and simulated experimentally (Williamson, 1990). Most studies focus on the most abundant chemicals, which may or may not be the most active chemical players. For conclusive proof of allelopathy, laboratory bioassays must demonstrate that the allelopathic effects can be attributed to the quantities of chemicals released under natural circumstances (Newman, 1978; Putnam and Tang, 1986; Inderjit, 1996). Finally, if population-level consequences of allelopathic interactions are of interest, interactions should be studied during the life history stages that are likely to have the largest demographic effects, and this consideration has focused research efforts on the sensitivity of seeds or seedlings. Allelopathic interactions that affect seed germination of a species either through inhibition or toxicity can occur both during seed-to-seed or plant-to-seed interactions. Seed-to-seed allelopathy, where one seed influences the germination of a co-occurring species (Gressel and Holm, 1964; Wardle et al., 1991; Van Staden and Grobbelaar, 1995; Laterra and Bazzalo, 1999), and plant-to-seed allelopathy, where an established plant influences the underlying seed bank, could alter the plant community structure prior to any resource competition. Complete inhibition is not essential for ecological significane, as delaying or slowing growth can also significantly hinder the competitive abilities of the affected plant and have large fitness effects.

Sagebrush is widely recognized as a species with allelopathic tendencies. Over 250 species of sagebrush (*Artemisia L.*) are distributed throughout the world (Güvenalp et al., 1998), and several studies have reported allelopathic activity in the genus *Artemisia* (Robertson, 1947; Reid, 1964; Friedman et al., 1977; Hoffman and Hazlett, 1977; Kelsey et al., 1978), identifying (possible) germination and growth inhibitors such as artemisinin (Duke et al., 1987, 1988; Chen and Leather, 1990; DiTomaso and Duke, 1991) and several monoterpenes (Weaver and Klarich, 1976). Among the volatiles credited with the inhibitory effects are the major constituents

in sagebrush emissions: camphor and the cineole epimers. Seed germination of a weedy monocot, *Echinochloa crusgalli*, and dicot, *Cassia obtusifolia*, was inhibited by 1,8-cineole, while root growth of *Cucumis sativa* (Muller, 1966) and shoot growth of *E. crusgalli* (Romagni et al., 2000) were reduced by applications of camphor and 1,4-cineole, respectively.

Artemisia tridentata (*Nutt.*) is one of the most widespread and economically important shrubs in North America (Freeman et al., 1991) and the dominant shrub over much of the semiarid western North America, occurring over a wide range of habitat types (Shultz, 1986). For purposes of optimizing grazing potential in western rangelands, there is considerable interest in understanding the interactions between sagebrush, a shrub with low forage value, and grasses and other species with greater forage value (Robertson, 1947). Muller (1966) suggested that in the Great Basin Desert, *A. tridentata* uses allelopathy to dominate in relationships with other species. *Nicotiana attenuata* Torr. ex Wats. (Solanaceae) is a wild tobacco plant also native to the Great Basin Desert (Wells, 1959) and is found in juniper–sagebrush habitats for 2–3 yr immediately following fires. Dormant seeds of *N. attenuata* germinate in response to a combustion product of cellulose (Baldwin et al., 1994) and the removal of nontoxic negative component(s) that suppress germination and are found in the litter of the dominant species that comprise the mature plant communities, including *A. tridentata* ssp. *tridentata* (Preston and Baldwin, 1999). While the specific chemical components that function as the positive germination signals have yet to be identified, the two component control over germination allows this species to time its germination with the postfire environment and locate its germination niche (Preston and Baldwin, 1999).

Since *N. attenuata* plants respond to MeJA, it is also possible that *N. attenuata* seeds may also be sensitive to this biologically active substance and provide a mechanism for sagebrush to interact with the seed bank underneath its foliage. Germination of cocklebur (*Xanthium pennsylvanicum* Wallr.) (Nojavan-Asghari and Ishizawa, 1998), lettuce (Yamane et al., 1981), sunflower (Corbineau et al., 1988, 1989), amaranth (Kepczynski et al., 1999), rapeseed and flax (Wilen et al., 1991) seeds are inhibited by MeJA application. However, all but cocklebur required concentrations $>100\mu$M, levels that may be physiologically irrelevant (Sembdner and Parthier, 1993). Applications of JA and MeJA appear to inhibit the germination of nondormant seeds (e.g., *Quercus robur*;) (Finch-Savage et al., 1996) and stimulate the germination of dormant seeds (e.g., *Acer tataricum*;) (Berestetzky et al., 1991; Daletskaya and Sembner, 1989; Creelman and Mullet, 1997). However, it is not clear if MeJA acts on the dormancy state or if this is a species-specific response. Methyl jasmonate has not been considered in previous allelopathic studies involving *A. tridentata*, probably because it is a minor component within the complex aroma of sagebrush (Preston et al., 2001). It is unlikely that MeJA is the sole chemical responsible for germination inhibition of *N. attenuata* by all later successional species of its habitat (Preston and Baldwin, 1999) since it is not found

in the inhibitory water extracts produced from juniper (*Juniperus osteosperma*) litter, another dominant later successional species (B. Krock, *unpublished data*). However, water extracts produced from sagebrush litter are significantly better at inhibiting *N. attenuata* germination as compared to other species' extracts (Preston and Baldwin, 1999). These results may be explained by MeJA acting in concert with the negative germination signal.

In the laboratory, *N. attenuata* plants delayed in germination have reduced competitive abilities and produce significantly fewer seeds (van Dam and Baldwin, 2001). Small delays in the laboratory bioassays can translate to significant delays in the field (Preston and Baldwin, 1999), causing *N. attenuata* to miss its germination window in the spring. Using field-based experiments, we first determined if tobacco seeds are affected by exposure to natural populations of sagebrush or soil collected near sagebrush. In the laboratory and under more controlled growth room conditions, we examined seed–seed and seed–seedling interactions between these species. Using a range of MeJA concentrations, we determined the sensitivities of *N. attenuata* and *A. tridentata* seeds. To gain a selective advantage from producing allelotoxins, a species must inhibit its potential competitors more than it inhibits itself (Williamson, 1990) and, therefore, we predict that *A. tridentata* will be less sensitive to MeJA. Because different genotypes of *N. attenuata* can vary in dormancy (dormant seeds germinate only after smoke exposure, while nondormant seeds germinate in response to water or nutrients), we examined if MeJA interacts with the seeds' dormancy state. Volatile transport is one pathway for MeJA to reach the seed bank, and we examined if volatile emissions from potted sagebrush plants and volatile MeJA-releasing devices affect the germination of *N. attenuata* seeds exposed for different lengths of time. To determine the proportion of germination inhibition that can be specifically attributed to MeJA, we manipulated the MeJA content of sagebrush chaff by heating, and serially diluting aqueous extracts of sagebrush litter and restoring the original concentrations of MeJA. Finally, we determined the generality of sagebrush's allelopathic effects by exposing seeds of a congeneric species of tobacco that co-occurs in the same habitat as *N. attenuata* and *A. tridentata*, namely *N. trigonophylla*. We exposed the two *Nicotiana* species that are sympatric with sagebrush, as well as five other allopatric *Nicotiana* species to water extracts of sagebrush litter and aqueous dilutions of MeJA.

METHODS AND MATERIALS

Species Description. *Artemisia tridentata* (Nutt.) is generally considered to be composed of four subspecies, *A. t.* ssp. *tridentata*, *A. t.* ssp. *vaseyana* (Rydb.) Beetle, *A. t.* ssp. *spiciformis* (Osterhout) Goodrich and McArthur, and *A. t.* ssp. *wyomingensis* Beetle and Young (Freeman et al., 1991). In this study, we focused on the subspecies *tridentata* and will herein refer to it as *A. tridentata* or sagebrush.

Sagebrush seeds for use in germination bioassays were manually separated from bulk chaff, defined here as a mixture of seeds, flower material, etc., from the floral meristem of the sagebrush plant (obtained from Goble Seed Co., P.O. Box 203, Gunnison, Utah, USA; lot ARTRVA-97). To obtain plants, chaff was sown onto potting soil and watered. The resulting seedlings were transferred to pots containing a 1:1 mixture of potting soil and sand that was supplemented with Osmocote 14:14:14 beads (Scotts Deutschland, Nordhorn, Germany) and micronutrients (Micromax, Scotts Deutschland). Plants were grown in a large growth room with a 16L:8D photoperiod and 27°C day–25°C night temperature cycle, 65% relative humidity with 1000–1500 μM m^2/sec PAR at plant growing level and watered with demineralized water when necessary.

Seeds of *N. attenuata* can be either nondormant [germinating in 9 d in response to water or nutrient (KNO_3) treatments] or dormant (germinating in 9 d in response to smoke treatments). Nondormant genotypes were originally obtained from natural populations in Utah, and the dormant genotypes originated from a population in Arizona. Seeds used were from these genotypes after propagation in the glass house.

Nicotiana trigonophylla is found in desert habitats of southern California and northern Mexico (Goodspeed, 1954) and occurs sporadically in washes in Utah and Arizona, but rarely in close proximity to *A. tridentata* (C.A. Preston and I.T. Baldwin, personal observation). It is not associated with the postfire environment (Wells, 1959) and is nondormant, germinating in response to applications of water or nutrients. Seeds originated from a single plant collection in 1990 in southwestern Utah (SUCS; T41S R17W section 34) and were combined with seeds collected from plants propagated in the glass house after one generation of selfing.

Nicotiana repanda Wildenow ex Lehmann occurs in northeastern Mexico and southern Texas, USA (Goodspeed, 1954). *N. benthamiana* is found in northern Australia's Great Sandy Desert and *N. sylvestris* Spegazzini et Comes occurs exclusively in northwestern Argentina (Goodspeed, 1954). *N. repanda*, *N. benthamiana*, and *N. sylvestris* seeds were originally obtained from the Oxford Tobacco Research Station (P.O. Box 1555, Oxford, North Carolina) and propagated for at least one generation of selfing in the glass house. *N. tabacum*, whose original distribution is obscured by human cultivation, is thought to have originated in northwestern Argentina (Goodspeed, 1954). *N. tabacum* L. cv. Xanthi-nc seeds carrying the N gene were obtained from Dr. Alexander J. Enyedi (Western Michigan University, Kalamazoo, Michigan, USA). *N. glauca* originated in Argentina but has spread widely to temperate regions around the world (Goodspeed, 1954), and seeds used were from a bulk collection of a wild population located near Riverside, California, USA.

Quantification of MeJA in Headspace. Emissions of MeJA were quantified by using methods previously described in Karban et al. (2000), with slight modifications. Briefly, to determine MeJA emissions from potted sagebrush plants, the

whole plant (young) or branches (mature) were enclosed in 1-l plastic containers, and traps were inserted into a hole in the side. To determine MeJA content of treated chaff, 400 mg of each chaff treatment was placed into individual 350-ml glass trapping containers with lids. An air inlet were positioned at the bottom of the chaff by inserting a tube leading from the lid to the bottom of the container. The trap was placed above the chaff through a second hole in the lid. The glass trapping containers containing samples were then placed into a 50°C water bath, and volatiles were collected. Using a diaphragm vacuum pump (model MOA P125 JH, Gast, Benton Harbor, Michigan, USA) pulling air from the headspace at 450 ml/min, volatiles were collected for 3–4 hr from the sagebrush plants and for 6 hr from the chaff treatments. Prior to trapping, 350 ng of triple ^{13}C-labeled MeJA (MW = 227) were added as an internal standard. Volatiles adsorbed on traps were eluted with 4 ml of dichloromethane (CH_2Cl_2) and gently concentrated to 100 μl before GC-MS analysis as described in Karban et al. (2000).

To quantify MeJA in water extracts of sagebrush litter, 10 ml of the aqueous solution were extracted twice with 10 ml CH_2Cl_2. Prior to extraction, 700 ng of $3\times^{13}$C-labeled MeJA were added as an internal standard to each replicate. The solvent was allowed to evaporate gently and the samples were reconstituted in 100 μl CH_2Cl_2 and transferred to a 150-μl glass insert and analyzed by GC-MS.

The *trans–cis* epimeric ratio in commercially available solutions of MeJA (MeJA 92:8 from Aldrich, Gillingham, Dorset, UK; MeJA 80:20 "extra epi" from Bedoukian Research, Danbury, Connecticut) was determined by GC-MS analysis.

Germination Bioassays. The germination inhibition activity of sagebrush, collected soils, sagebrush water extracts, and MeJA dilutions was determined using a previously described seed germination bioassay (Baldwin and Morse, 1994; Baldwin et al., 1994; Preston and Baldwin, 1999). Depending on the experiment, three replicate bioassays were performed in containers, each containing 10–20 *N. attenuata* seeds from nondormant or dormant genotypes. For *A. tridentata*, three or four replicate bioassay containers, each containing between 5-30 seeds were used. Seeds were placed on a thin layer (approximately 3 g) of sterile sand (Merck, Darmstadt, Germany). To determine maximum germination, each experiment also included three or four replicate water-control bioassays or nutrient-control bioassays, in which seeds were treated only with distilled water or 1 mM KNO_3, respectively. Seed cups were placed in a growth chamber (Snijders Scientific, Tilburg, Holland; model S 1084) with a 16L:8D photoperiod and 32°C day–27°C night temperature cycle. Seeds were examined after 72 hr and monitored daily for 9–43 d, depending on the experiment, for radicle emergence. To determine seed viability, 250 μl of 1 mM gibberellic acid$_3$ (GA_3; Duchefa, Haarlem, Netherlands) was applied on the final monitoring day to evaluate treatment effects (day varied with the experiment). Previous trials with *N. attenuata* seeds determined that all viable seeds germinate in response to this GA_3 treatment within 4 d (T. Krügel and C.A. Preston, unpublished data).

Burial of Seed Bags Adjacent to Sagebrush and Seed Germination on Soils Collected Near Sagebrush. In the Great Basin Desert of SW Utah, between-plant distances measured at two *N. attenuata* populations located in recently burned areas bordered by sagebrush populations showed that tobacco was not found closer than 50 cm to an unburned sagebrush (C.A. Preston, unpublished data). Since plants of *N. attenuata* rarely grow near sagebrush plants in the field, this may suggest that components released by sagebrush are inhibiting germination of tobacco seeds from the underlying seed bank. To determine if exposure to sagebrush affected germination of *N. attenuata* seeds residing in the underlying seed bank, we buried bags of seeds different distances from natural sagebrush plants for 4 months. Seed bags, each containing 80–100 nondormant *N. attenuata* seeds, were prepared using plastic "no-see-um" netting (Tent City, Buffalo, New York, USA). The seed bags were buried on February 29, 2000, at a single sagebrush population (Pahcoon site, T41S R18W section 10 in Utah). Twelve sagebrush plants located along the edge of the population were selected, all of approximately the same size and healthy appearance. At each plant, two seed bags, one sealed in a small plastic bag, were buried at four locations along a linear transect leading out of the sagebrush population and into an open area. Seed bags were buried near to the base of the sagebrush, at the edge of the sagebrush canopy, 20 cm from the canopy, and 40 cm from the canopy (locations 1, 2, 3, and 4, respectively; Figure 1, left inset). On June 27, 2000, the seed bags were recovered and brought back to the lab in individual small plastic bags (with the plastic and net bag from each burial site kept together) containing a small amount of the soil from their respective burial sites. A germination bioassay was performed as described above with three replicates of 20 *N. attenuata* seeds from each bag at each site.

To determine if components released by sagebrush could accumulate in the soil surrounding the sagebrush plant in sufficient quantities to affect germination of exposed *N. attenuata* seeds, even when the sagebrush plant was no longer present, we collected soil from different proximities to a population of sagebrush. The underlying A_1 soil horizon was collected at a single *A. tridentata* population (Pahcoon site, T41S R18W section 10) in Utah from directly underneath sagebrush plants (10 replicates), between sagebrush plants (10 replicates) and 10 m outside of the sagebrush population (5 replicates). Soils were brought back to the laboratory, and the general bioassay procedure was followed, except that 1.5 cm^3 of soil was used instead of the sand. Twenty nondormant *N. attenuata* seeds were placed directly on top of the soil, and the soil was saturated with 1 mM KNO_3 to eliminate the possibility that germination responses resulted from nutrient differences between the soil collections. Germination was monitored for 8 d.

Germination of N. attenuata Seeds Exposed to A. tridentath Seeds or Seedlings. To determine if sagebrush seeds or seedlings could influence germination of *N. attenuata* seeds occupying the same seed bank, we combined 10 nondormant *N. attenuata* seeds with 5–30 *A. tridentata* seeds or 5–30 *A. tridentata* 2-to 3-d-old

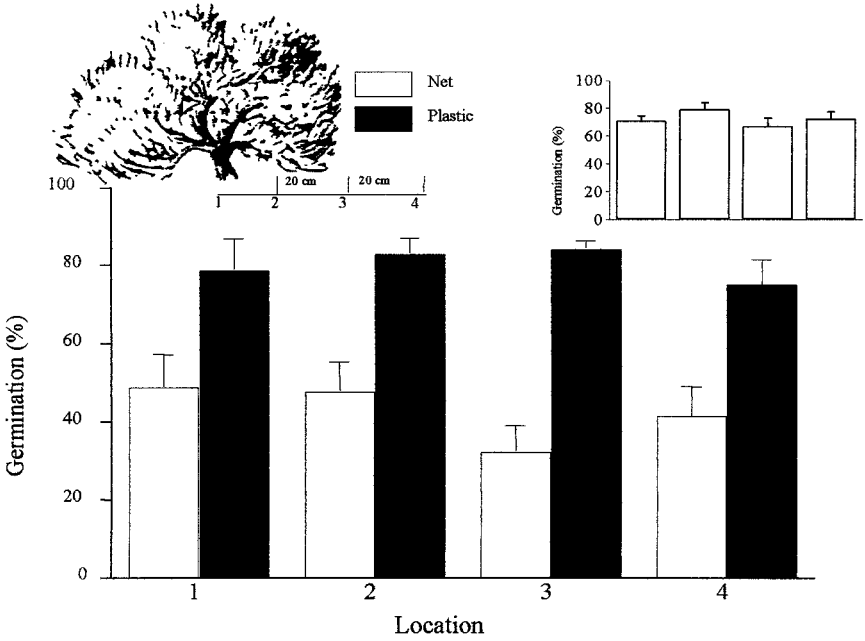


FIG. 1. Mean (+ 1 SEM) seed germination percentages at day 9 and day 16 (inset) of *Nicotiana attenuata* seeds enclosed in bags made of netting (and hence exposed to soil constituents; clear bar) or bags made of netting placed into plastic bags (and hence protected; black bar) and buried for 4 months at different distances from *Artemisia tridentata* ssp. *tridentata* plants in SW Utah. Two seed bags, one exposed and one protected, were buried at four locations in a linear transect from sagebrush plants: (1) directly adjacent to sagebrush trunk; (2) at the edge of the sagebrush canopy; and (3) 20 cm; and (4) 40 cm from the edge of the sagebrush canopy. This burial scheme was repeated using 12 sagebrush plants located along the edges of a single population and the linear transects led into an open area to avoid interference from other sagebrush plants.

seedlings in the seed germination bioassay container. Germination of *N. attenuata* seeds was monitored for 9 d.

Effects of Two Naturally Occurring MeJA Epimers on N. attenuata and A. tridentata Germination. The *cis* epimer of MeJA is thought to be the more biologically active epimer (Beale and Ward, 1998) and the one that may be the airborne signal eliciting herbivore resistance in *N. attenuata* plants growing adjacent to damaged sagebrush in nature (Karban et al., 2000; Preston et al., 2001). To test the sensitivity of *N. attenuata* and *A. tridentata* seeds to the two epimers, we applied a range of quantities of the two epimers and monitored germination. Dilutions of MeJA were made by weighing 2.24 g of MeJA [*trans (trans–cis* ratio, 92:8), Aldrich, Gillingham, Dorset, UK; *cis* (*trans–cis* ratio, 80:20), "extra-epi" MeJA,

Bedoukian Research, Danbury, Connecticut] and adding 10 ml of 1 mM KNO_3 to produce a 1 M MeJA solution. The solution was sonicated and serial dilutions were made by adding 1 ml of the MeJA solution to 9 ml of 1 mM KNO_3. This was repeated to produce solutions in the range of 10^{-2}–10^{-8} M MeJA in 1 mM KNO_3 (see *X* axis of Figure 3 below for amounts of the compounds added to each seed cup). Since preliminary experiments suggested that *A. tridentata* seeds were less sensitive to MeJA application (e.g., more MeJA was required to inhibit germination), MeJA solutions were applied in the range of 10^{-2}–10^{-6} M. Moreover, germination of *A. tridentata* seeds was more variable; hence, four replicate seed cup bioassays were used. In summary, the experiment contained 170 seed cup bioassays in total: 87 *N. attenuata* bioassays with 20 seeds each (2 epimers MeJA $\times$ 7 incremental M solutions $\times$ 2 volumes $\times$ 3 replicates + 3 nutrient-controls) and 83 *A. tridentata* bioassays with 20 seeds each (2 epimers MeJA $\times$ 5 incremental M solutions $\times$ 2 volumes $\times$ 4 replicates + 3 nutrient-controls). The seeds were treated with 0.5 ml of the MeJA solutions and additionally received 1 ml of 1 mM KNO_3 to equalize nutrient levels among all seed cups.

Effect of MeJA on Dormant and Nondormant N. attenuata Seeds. Evidence from several plant species suggests that the effects of MeJA depend on the dormancy state of the seeds. Dormant seeds are stimulated while nondormant seeds tend to be inhibited (Creelman and Mullet, 1997). To determine if this pattern applies to *N. attenuata* seeds, we used two different genotypes, one dormant and one nondormant. Aqueous solutions of MeJA were made in a range of 10^{-3}–10^{-6} M (see previous section). The dormant seeds were treated either with the MeJA dilutions or exposed for 1 hr to a 1:300 dilution of liquid smoke [smoke: water (v/v); House of Herbs, Passaic, New Jersey, USA], that was then removed prior to application of the MeJA treatments. MeJA may stimulate dormant seeds to germinate in the absence of the smoke-derived germination signal, or germination may be affected when seeds have received the signal for germination (and hence ending their dormancy), either by increasing or decreasing the rate of germination.

To determine background and maximum germination, dormant seeds were treated with nutrients or the smoke solution followed by nutrients, respectively. Nondormant seeds were treated only with the MeJA dilutions, and controls were treated with nutrients to determine the maximum germination response. The experiment included 81 seed cup bioassays, with 54 replicate seed cup bioassays containing dormant seeds (2 treatments x 4 incremental M solutions $\times$ 2 volumes $\times$ 3 replicates + 3 nutrient-controls + 3 smoke-controls) and 27 replicate seed cup bioassays with nondormant seeds (1 treatment $\times$ 4 incremental M solutions $\times$ 2 volumes $\times$ 3 replicates + 3 nutrient-controls).

Effect on N. attenuata Germination of Exposure to MeJA and Sagebrush Volatiles. To determine if volatiles released by sagebrush and volatile MeJA could inhibit germination of *N. attenuata* seeds, we placed seed cups downwind of either potted sagebrush plants or MeJA-releasing devices. Twenty-four seed cups (12

with and 12 without lids) each containing 20 nondormant *N. attenuata* seeds on a thin layer of sand were placed into transparent plastic 18.5-l growth chambers (Rubbermaid; Wooster, Ohio) and covered with a piece of UV transparent Plexiglas (UV-T). Airflow was controlled with a fan (7 cm diam.) attached to the outside of each chamber, which pulled air through five small holes drilled on the opposite side of the growth chamber. Fan speed was regulated by a rheostat and used to circulate air through the growth chamber, with the seed cup bioassays located downwind of two potted, young sagebrush plants or a MeJA-releasing device, either releasing MeJA at a low (3.5 μg/hr or high (5 μg/hr) rate. For these devices, we used crimped-top 1.5-ml glass vials with Teflon inner linings containing 0.5 ml MeJA. Changes in the mass of the devices were used to determine MeJA emission rates. Similarly, 24 seed cup bioassays each containing 20 *N. attenuata* seeds on a thin layer of sand, 12 with and 12 without lids, were placed into a large growth chamber with 20 individually potted, mature sagebrush plants. Seed cups were distributed underneath the sagebrush plants. Six seed cups (3 with and 3 without lids) were haphazardly selected and removed 7, 14, 21, or 28 d after being enclosed into the small or large growth chambers. After removal, the seed cups were given sufficient amounts of 1 mM KNO_3 to saturate the sand and placed into a germination chamber. Germination was monitored for 9 d. Emission of MeJA by the sagebrush plants was quantified by volatile trapping of similar sagebrush plants (same age and size).

Germination of N. attenuata on Chaff: Manipulating MeJA Content by Heating. Significant amounts of MeJA were found in the chaff collected from *A. tridentata* plants in natural populations. Using aqueous extraction of three 1-g samples, the chaff was determined to contain approximately 2.57 $\pm$ 0.05 μg MeJA/g. The content of MeJA in the chaff was experimentally manipulated by heating it in an oven for 3 hr at 100°C or for 1 or 3 hr at 150°C to volatilize the MeJA. The amount of MeJA remaining in each chaff treatment was determined by trapping MeJA from the headspace of each sample (see the *X* axis of Figure 6 for rates of MeJA release). A layer of baked or unbaked chaff (approximately 0.1 g) was placed in a seed cup underneath a thin layer of sand, on top of which 10 nondormant or 10 dormant *N. attenuata* seeds were placed, with three replicate seed cup bioassays for each treatment. The dormant *N. attenuata* genotype was used to determine if heating produced substances similar to those found in smoke, which stimulate germination of *N. attenuata*. Two milliliters of 1 mM KNO_3 were added to each seed cup to saturate the chaff and sand layers. Germinated *N. attenuata* seeds were counted after 72 hr and then periodically until day 43. At the end of this period, seeds from each replicate within each treatment that had not yet germinated were removed, washed in distilled water, and transferred to a single seed cup containing only sand. Each seed cup received 1 ml of 1 mM KNO_3 and 500 μl of 1 mM GA_3 to determine the viability of the remaining seeds.

Effects of Water Extracts of Sagebrush Litter and MeJA on a Co-Occurring Congeneric Tobacco. In a previous study investigating positive and negative germination signals for *N. attenuata*, germination of *N. trigonophylla*, on a congeneric species of tobacco that co-occurs in similar habitats as *N. attenuata*, was not inhibited by water extracts produced from juniper litter while *N. attenuata* germination was completely inhibited (Preston and Baldwin, 1999). To determine if water extracts of sagebrush litter and MeJA affected germination of *N. trigonophylla* seeds, we treated seeds with aqueous dilutions of MeJA and extracts of sagebrush litter. For comparison, the treatments were also applied to nondormant *N. attenuata* seeds. Three seed cups for each species were treated with only 1 mM KNO_3 or distilled water as a control. The same sagebrush water extracts and dilutions were used as in the *N. attenuata* MeJA addition experiment, and three cups were treated with only distilled water, as controls.

Can Addition of MeJA to Diluted, Inactive Sagebrush Water Extract Restore Germination Inhibition? Given that sagebrush water extracts are chemically complex, containing many substances that might affect seed germination in addition to MeJA, it is important to determine the proportion of the germination inhibition that can be attributed to MeJA. We determined the MeJA concentration in a water extract of sagebrush litter known to completely inhibit germination of *N. attenuata* seeds, serially diluted this extract until it was no longer inhibitory, and added the appropriate amounts of MeJA to the diluted extracts to restore the amount of MeJA that was present in the original extract (Figure 7C inset). To prepare the extract, we collected the litter-containing A_0 horizon from underneath sagebrush plants at a single population (Apex Mine site, T43S R18W section 36) in Utah and stored it at -20°C until extracted. Seventy-five grams of litter was placed in a 2-l glass Erlenmeyer flask with 1.5 l of distilled water and stirred overnight at room temperature. The aqueous phase was transferred to 50-ml centrifuge tubes and centrifuged in an Avanti J-25 centrifuge (Beckman, Palo Alto, California, USA) at 12,000 rpm for 15 min at 10°C, and the supernatant was used immediately for MeJA quantification and in seed germination bioassays. The sagebrush litter water extract was applied undiluted or diluted with distilled water to produce a final dilution series of (sagebrush litter water extract–water, v/v): 1:0, 1:1, 1:4, 1:9, 1:14, 1:19, 1:24. As a control, three seed cup bioassays were treated only with water (see 0:1 on the *X* axis of Figure 7A,C). The concentration of MeJA was determined by extracting three replicates of 10 ml of undiluted sagebrush litter water extract. Within the samples, there was 195.97 $\pm$ 23.65 ng MeJA/ml. For this germination assay, we calculated the additions of MeJA based on 200 ng/ml. A dilution of MeJA was made by weighing 1.0 mg of MeJA into 10 ml CH_2Cl_2 and distributed to 20-ml glass scintillation vials as required for each dilution. The solvent was allowed to evaporate, and an appropriate amount of sagebrush litter water extract was added. The vials were sonicated to distribute the MeJA and shaken vigorously prior to application to the seeds.

Does MeJA Inhibit Germination of Other Tobacco Species That Do Not Co-Occur with Sagebrush? Given that both *N. attenuata* and *N. trigonophylla* are sensitive to MeJA, we examined if the sensitivity of MeJA is due to the specific habitat in which they co-occur, namely containing sagebrush, or if it is a more general species-specific response. Dilutions of MeJA were made as described in a previous section.

Effects of the Two Naturally Occurring MeJA Epimers on N. attenuata and A. tridentata. Germination were examined, with the exception that distilled water was used instead of 1 mM KNO_3. Serial dilutions were made to produce final aqueous solutions in the range of 10^{-1}–10^{-10} M MeJA. Controls were treated with water only to determine the maximum germination response within each species. All treatments were applied as 1-ml aliquots per seed cup. *Nicotiana* species had three replicate seed cup bioassays for each treatment, and *Artemisia* had five replicate seed cup bioassays for each treatment due to its more variable response. In summary, the experiment included 286 seed cup bioassays, with 231 seed cup bioassays containing *Nicotiana* species [7 species $\times$ 11 incremental M solutions (including water-control) $\times$ 3 replicates] and 55 seed cup bioassays containing *A. tridentata* [1 species $\times$ 11 incremental M solutions (including water-control) $\times$ 5 replicates].

Statistical Analyses. The percentage of all seeds germinating in each bioassay container at each observation was arcsine-transformed to meet the assumptions of normality for the statistical tests. Repeated-measures ANOVAs on the transformed percentages were used to analyze main effects. One-way ANOVAs were used to evaluate differences on the treatments on individual days. Fisher's PLSD *post hoc* tests were performed on data from the final day of the experiments (prior to GA_3 addition) to compare individual treatments. Analyses were performed with the STATVIEW 5.0 statistical package (Abacus Concepts, Berkeley, California).

RESULTS

Burial of Seed Bags and Seed Germination in Soils Collected Near Sagebrush. Seeds in seed bags exposed to soil had delayed germination compared to seeds in seed bags that were in protective plastic bags (Figure 1). However, there were no significant differences in germination rate or final germination percentages among the different locations for seeds in either the unprotected (net; repeated measures ANOVA $F_{18,240} = 0.972$, $P = 0.49$; ANOVA on day 9, $F_{3,40} = 0.864$, $P = 0.47$) or protected bags (plastic; repeated measures ANOVA $F_{18,240} = 1.090$, $P = 0.36$; ANOVA on day 9, $F_{3,40} = 1.574$, $P = 0.21$). Enclosure in plastic bags clearly reduced or eliminated exposure to sagebrush allelochemicals, but alternative explanations for the germination delay should be considered. Plastic bags may have altered the seeds' microenvironment, such as temperature, humidity, and

exposure to soil microbes, and these alterations may account for the germination delay.

To standardize microclimate exposure, we examined the germination of naive seeds on soils collected at different locations in relation to sagebrush. Seeds on soil collected from underneath sagebrush showed less germination than seeds placed on soil collected from between sagebrush plants or outside of the sagebrush population (Figure 2; one-way ANOVA $F_{2,22} = 13.541$, $P = 0.001$). *Post-hoc* analysis revealed that soil collections from between and outside were different from under ($P's < 0.001$) but not different from each other ($P = 0.99$). We conclude that germination of *N. attenuata* seeds can be affected by unknown factors that may be present in the soil from the sagebrush.

Germination of N. attenuata Seeds Exposed to A. tridentata Seeds or Seedlings. The final germination percentages of *N. attenuata* seeds exposed to different numbers of *A. tridentata* seeds or seedlings were not significantly different (one-way ANOVAs: $F_{5,24} = 1.879$, $P = 0.14$ and $F_{3,11} = 2.466$, $P = 0.12$, respectively). Likewise, repeated measures ANOVAs found no differences among the rates of germination (seeds: $F_{30,144} < 0.1$, $P < 0.001$; seedlings: $F_{18,66} = 1.525$, $P = 0.11$). We conclude that neither seed–seed competition nor seed–seedling allelochemically mediated interactions are likely to be important determinants of *N. attenuata*'s seed bank dynamics.

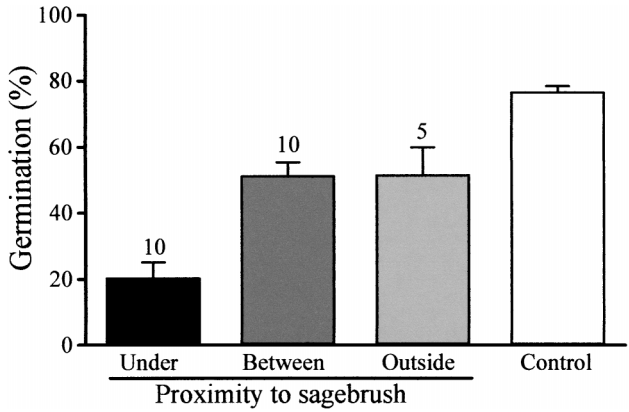


FIG. 2. Mean (+ 1 SEM) seed germination percentages at day 8 for seeds placed on 2 cm^3 of the A_1 soil layer (without litter) from directly under sagebrush plants (black bar), between sagebrush plants (dark gray bar), and approximately 10 m from the sagebrush stand (light gray bar). Seeds germinating on sand served as a control (clear bar). Values above the bars indicate the number of soil collections tested for each site; three replicate seed germination bioassays were used for each soil collection.

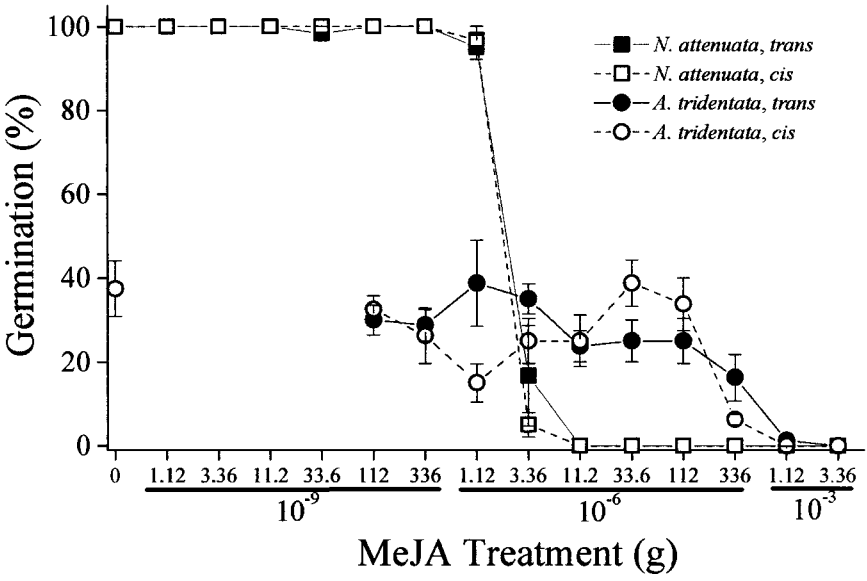


FIG. 3. Mean (±1 SEM) percentage germination response at day 8 for *N. attenuata* (squares) and *A. tridentata* (circles) seeds exposed to increasing quantities of trans- (solid symbols) and *cis*-MeJA (white symbols). The x-axis indicates amount of MeJA (1.12 ng to 3.36 mg/seed cup) applied to the 20 seeds in each bioassay. For each treatment, three replicate bioassays were used for *N. attenuata* seeds and four for *A. tridentata* seeds.

Effects of MeJA Epimers on N. attenuata and A. tridentata Germination. There was no difference between either species in response to *trans*- and *cis*-MeJA (Figure 3; *N. attenuata*: $F_{1,82} = 1.341 \times 10^{-6}$, $P = 0.99$; *A. tridentata*: $F_{1,78} = 0.385$, $P = 0.54$), and the replicates within the *trans* and *cis* treatments were combined for each MeJA amount to increase statistical power for our subsequent analyses. Compared to nutrient-controls, germination was significantly ($P < 0.001$) inhibited in *A. tridentata* when 20 seeds in a seed cup were exposed to 336 μg MeJA (16.8 μg/seed) but in *N. attenuata*, 1.12 μg MeJA was inhibitory for 20 seeds in a seed cup (0.056 μg/seed; $P = 0.016$). The greater tolerance of *A. tridentata* is consistent with predictions for the allelopathic use of a germination inhibitor: the releaser must be less sensitive to the allelochemical than the receiver.

Effect of MeJA on Dormant and Nondormant N. attenuata Seeds. MeJA inhibited the germination of both dormant and nondormant seeds of *N. attenuata*, but dormant seeds were more sensitive than nondormant seeds (Figure 4). Dormant seeds when treated with either water or smoke germinated significantly less when exposed to a range of MeJA quantities (one-way ANOVAs: $F_{8,18} = 2.569$, $P = 0.046$ and $F_{8,18} = 39.107$, $P < 0.001$, respectively). Dormant and nondormant

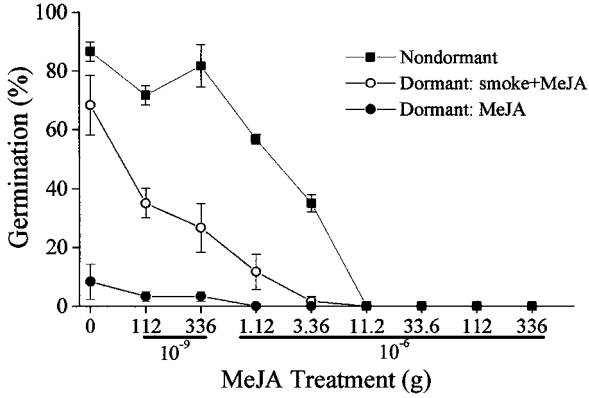


FIG. 4. Mean (±1 SEM) percentage germination response on day 8 for nondormant (squares) and dormant (circles) genotypes of *N. attenuata* seeds to a range of MeJA quantities. Dormant genotypes require exposure to a component of smoke before they initiate germination. Dormant seeds were treated with MeJA either with (open) smoke factors or without (solid) to determine if MeJA might function as a stimulant for the dormant genotypes. Each treatment consisted of three replicate bioassays of 20 seeds each.

N. attenuata seeds had different sensitivities to MeJA (two-way ANOVA $F_{7,32} = 12.705$, $P < 0.001$). *Post-hoc* analyses revealed that the germination of dormant seeds pretreated with smoke was inhibited ($P = 0.004$) below that of smoke-controls when seed cups were exposed to 112 ng MeJA (5.6 ng/seed). However, nondormant seeds revealed a more variable response with significant inhibition at 112 ng ($P = 0.005$) but not at 336 ng ($P = 0.37$) per seed cup (5.6 and 16.8 ng/seed, respectively) and inhibition again at all higher exposures ($P \leq 0.032$). Germination of nondormant seeds in a previous experiment (Figure 4) was inhibited with 3.36 μg MeJA per seed cup (168 ng/seed). Although we cannot directly compare the inhibition amounts between the experiments, it is interesting to note that each of these tested seeds were from different collection sites in Utah. This suggests that the variation between dormant and nondormant lines may be due to differences among genotypes rather than differences in their dormancy state. We conclude that the germination of *N. attenuata* seeds is inhibited by the presence of MeJA, regardless of their state of dormancy.

Effect on Seed Germination of Exposure to Sagebrush and MeJA Volatiles. The environments in this experiment were not replicated and, therefore, we could not compare germination responses between the different environments, but only within an environment (uncovered versus covered). Exposure to MeJA and sagebrush volatiles delayed germination (Figure 5, delays in germination determined by one-way ANOVAs performed on each day to compare transformed germination percentages between covered and uncovered seed cups harvested simultaneously,

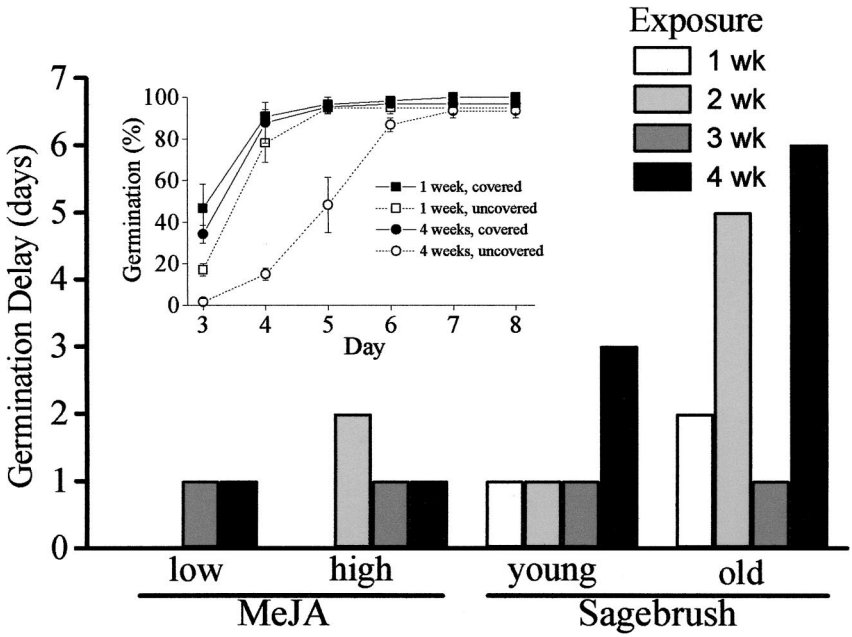


FIG. 5. Magnitude of statistically significant delays in germination of seeds that were exposed for different lengths of time to volatile MeJA or airborne volatiles released from mature or young sagebrush plants. Uncovered seed cups containing 20 *N. attenuata* seeds on dry sand were placed into small growth chambers with either MeJA-releasing devices emitting MeJA at a low (3.5 μg/hr) or high (5.0 μg/hr) rates, two small sagebrush plants (young) or nothing (control). In a large growth chamber, similar seed cups, with or without covers, were haphazardly placed underneath mature sagebrush plants (old). For seed cups with equivalent exposure periods, significant delays from controls were determined with one-way ANOVAs on germination rates on days 3–8. Three replicate germination bioassay containers from each treatment and under each age of sagebrush were removed at weekly intervals. Inset depicts the mean ($\pm$1 SEM) percentage germination response for the seed cups with (solid) or without (open) covers exposed to the volatile emissions of the mature sagebrush plants for 1 wk (squares) or 4 wk (circles) to illustrate how the germination delays were determined.

$Ps < 0.05$). Under our experimental conditions in the laboratory, young sagebrush plants released 8 ng/g/hr plant. Each sagebrush plant in the small growth chamber had an approximate foliage mass of 200 g, indicating that combined MeJA levels from both plants were approximately 3.2 μg/hr. MeJA was released from the releasing devices at low and high rates (3.5 and 5 μg/hr, respectively). The mature sagebrush in the large growth chamber released approximately 2 ng/g/hr. Each

mature sagebrush plant had an approximate foliage mass of 400 g, indicating the total MeJA emission from all mature plants in the large growth chamber was approximately 16 μg/hr. Both volatile MeJA and emissions from sagebrush delayed germination similarly, and the delays appeared to correlate positively with the quantities of MeJA being released. Germination delays were longer in seeds sharing growth chamber space with the mature sagebrush or the high MeJA-releasing device than for seeds sharing growth chamber space with the young sagebrush plants or the low MeJA-releasing device.

Germination of N. attenuata on Chaff; Manipulating MeJA Content by Heating. Heating the chaff to 150°C did not stimulate germination by producing the positive cue; germination of dormant seeds was inhibited by exposure to the heated chaff compared to smoke-treated controls (data not shown; one-way ANOVA $F_{5,12} = 16.731$, $P < 0.001$). *Post-hoc* comparisons revealed that germination of seeds exposed to each chaff treatment was less than in the smoke-controls (Ps < 0.001) and not significantly different than in nutrient-controls (Ps ≤ 0.07). The germination rates in the unheated and the 100°C treatments) were not significantly different from each other ($P = 0.24$) but were less than that of the 150°C treatments ($P < 0.001$). At 150°C, the germination in the 1- and 3-hr heating intervals were not different ($P > 0.39$). The germination response correlated negatively with the quantity of MeJA volatilized from the heat-treated chaff. The amount of MeJA trapped from the headspace represents the amount of MeJA present in the chaff samples (i.e., the lower amounts of MeJA trapped indicate lower quantities of MeJA present in the chaff). Hence, the lower the MeJA emission, the lower the content of MeJA in the chaff, the greater the germination response. After removing the seeds from their respective chaff environments, a high percentage of germination was achieved in each treatment (Figure 6 inset), demonstrating that germination was inhibited by chaff exposure but it was not toxic to the seeds.

Effects of Water Extracts of Sagebrush Litter and MeJA on a Co-Occurring Congeneric Tobacco. Seeds of *N. trigonophylla* responded differently to water extracts of sagebrush litter than did *N. attenuata* seeds (Figure 7A; two-way ANOVA $F_{7,32} = 11.559$, $P < 0.001$). A repeated measures ANOVA revealed that *N. trigonophylla*'s germination rate differed among the dilutions ($F_{35,80} = 4.606$, $P < 0.001$). Seeds treated with the undiluted sagebrush litter water extract germinated slower than all other dilutions (Ps ≤ 0.019), except for the 1:1 dilution ($P = 0.058$), which additionally germinated slower than the 1:14 dilution ($P = 0.041$). *Post-hoc* analyses on final germination percentages on day 8 revealed that *N. trigonophylla* seeds were not inhibited by any dilution of water extract of sagebrush litter (Ps ≤ 0.074) when compared to the water-controls. However, seeds treated with a dilution of 1:14 (sagebrush extract: water, v/v) germinated more than those in the water-control ($P = 0.045$). In contrast, *N. attenuata* seeds were inhibited at all dilutions (Ps ≤ 0.003).

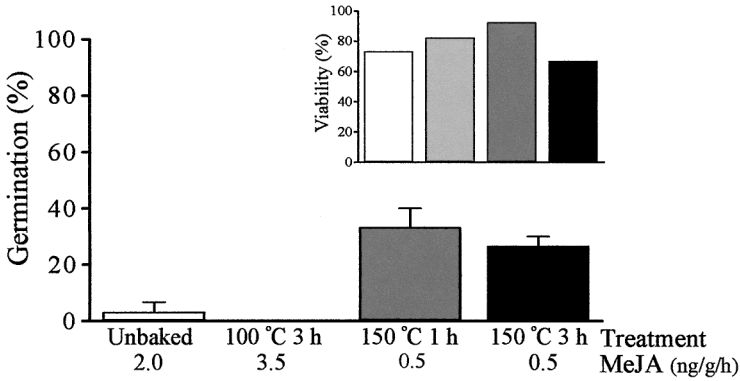


FIG. 6. Mean (+ 1 SEM) germination on day 43 for *N. attenuata* seeds exposed to sagebrush litter or sagebrush litter that had been heated at 100°C for 3 hr (light gray bar), 150°C for 1 hr (dark gray bar), or 150°C for 3 hr (black bar). Each treatment had three replicate bioassay containers, each with 20 seeds. The amount of MeJA released from litter after heating (reflecting the amount of MeJA remaining in the chaff) is shown below the x axis. The inset depicts the viability of all seeds removed from the three replicates of each treatment, determined after seeds were rinsed with water and treated with gibberellic acid (GA_3).

Similarly, *N. trigonophylla* and *N. attenuata* seeds showed different sensitivities to MeJA treatment (Figure 7B; two-way ANOVA on day 8, $F_{8,36} = 9.914$, $P < 0.001$). *Post-hoc* comparisons revealed that the germination of both species was inhibited by the application of 1.12 μg MeJA per seed cup (56 ng/seed) as compared to nutrient-controls (*N. trigonophylla* $P < 0.001$, *N. attenuata* $P = 0.037$). To compare each species for each MeJA treatment, we standardized the germination numbers for day 8 by making them relative to the control by subtracting the germination percentages for each replicate in each treatment from the average germination in their respective nutrient controls and dividing by their respective nutrient-control average. *Post-hoc* analyses revealed that *N. trigonophylla* was more inhibited than *N. attenuata* when seeds were treated with 1.12 (56 ng/seed) and 3.36 μg MeJA (168 ng/seed; Ps ≤ 0.048).

We conclude that germination of nondormant seeds from the co-occurring tobacco, *N. trigonophylla*, can be delayed by water extracts of sagebrush litter but not inhibited. This is in contrast to *N. attenuata*, whose germination can be completely inhibited by the same treatments. While in this experiment it appears that *N. trigonophylla* is more sensitive to MeJA than *N. attenuata*, we can only conclude that both species are sensitive. The variation in the germination response of *N. attenuata* seeds exposed to dilutions of MeJA, possibly due to genotypic variation, makes this experiment insufficient to demonstrate fine-grained differential sensitivities between the two congeneric tobaccos.

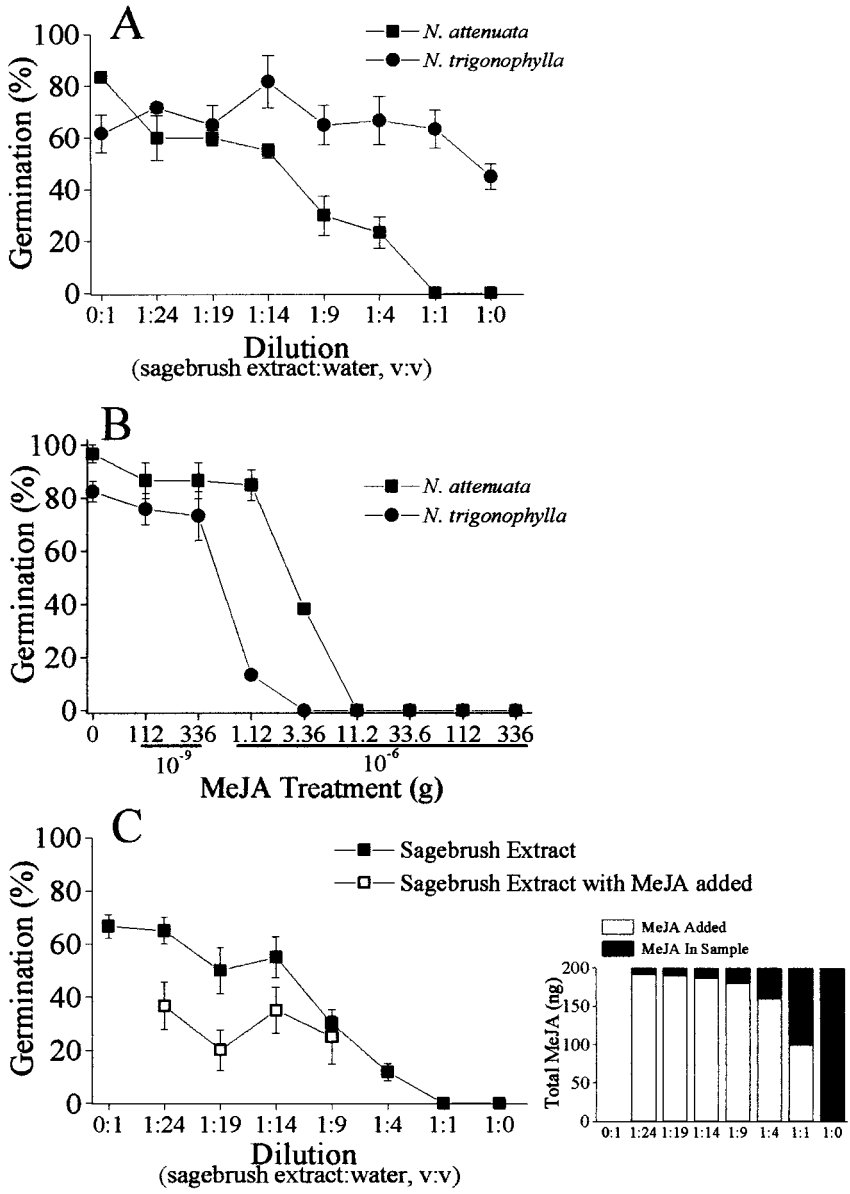


FIG. 7. Mean (±1 SEM) percentage germination response on day 8 for *N. attenuata* (squares) and its nondormant conspecific, *N. trigonophylla* (circles) to (A) dilutions of the water extract produced from intact sagebrush litter, (B) dilutions of MeJA, and (C) dilutions of water extract produced from intact sagebrush litter alone (solid symbols) or

Can Addition of MeJA to Diluted, Inactive Sagebrush Water Extract Restore Germination Inhibition? By diluting the sagebrush extract, we can decrease the inhibition of *N. attenuata* seeds. To determine if MeJA is an active allelopathic agent within the extract, we supplemented the dilutions with quantities of MeJA such that all dilutions had the same quantity of MeJA (200 ng/ml; Figure 7C inset). Water extract produced from sagebrush litter inhibited germination of *N. attenuata* seeds in all dilutions tested as compared to water-controls (Figure 7C, Ps < 0.001). This was also true of water extracts of sagebrush litter with additions of MeJA (Figure 7C, Ps < 0.001). A two-way ANOVA revealed differences between the sagebrush extract alone and sagebrush extracts with the addition of MeJA ($F_{1,16} = 13.621$, $P = 0.002$). However, the undiluted sagebrush extract inhibited germination more effectively than dilutions receiving additional MeJA to restore levels found in the undiluted sagebrush extract (one-way ANOVA $F_{4,10} = 7.730$, $P = 0.004$). Additionally, the 200 ng MeJA/ml (10 ng/seed) contained within the undiluted sagebrush extract is within the range of the quantity of MeJA that begins to affect germination of *N. attenuata* seeds treated with aqueous solutions of MeJA (Figure 5). Comparing the amount of inhibition resulting from the MeJA additions for each sagebrush extract dilution, we determined that 16.67–60% of the inhibitory activity of the original extract could be attributed to the MeJA. We conclude that although MeJA could account for a portion of the inhibitory activity of the extract, it is not the sole inhibitory component, and it likely acts in concert with other allelochemicals.

Does MeJA Inhibit Germination of Other Tobacco Species That Do Not Co-Occur with Sagebrush? Comparing seed germination responses on day 9 within each species, seeds of all tobacco species responded differently to the MeJA treatments (one-way ANOVAs $F_{10,22}$s ≤ 679.39, Ps < 0.001) as did the seeds of *A. tridentata* ($F_{10,44} = 10.95$, $P < 0.001$). *Post-hoc* analysis revealed differences in the amount of MeJA necessary to inhibit germination of each species below that of the water-controls. In this bioassay, *N. trigonophylla* was the most sensitive, with germination inhibited when 0.224 μg MeJA/seed cup (11.2 ng/seed) was applied. Both *N. attenuata* and *N. benthamiana* were inhibited by 22.4 μg MeJA/seed cup (1.12 μg/seed). *N. sylvestris* and *N. glauca* as well as *A. tridentata*

←

supplemented with sufficient quantities of MeJA to restore the MeJA content to levels found in the undiluted extract (open symbols). GC-MS quantification of MeJA contents in three replicate samples from the sagebrush litter water extract revealed that the extract contained 195.97 $\pm$ 23.65 ng/ml. The inset depicts the amount of MeJA added (open bar) to each dilution of the diluted sagebrush water extracts to supplement the MeJA in the sample (black bar), restoring the total MeJA content to 200 ng/ml. Each treatment had three replicate bioassays of 20 seeds.

seeds were inhibited by 224 μg MeJA/seed cup (11.2 μg/seed). *N. repanda* and *N. tabacum* were the least sensitive species, with germination inhibition at 2240 μg MeJA/seed cup (112 μg/seed). Although the quantity of MeJA required to inhibit germination of *N. attenuata* seeds was greater than in previous experiments, it is less than the amount required to inhibit *A. tridentata* germination. Tobacco species that do not co-occur with sagebrush are less sensitive to MeJA, with four of five nonassociated *Nicotiana* species requiring more MeJA to inhibit germination.

DISCUSSION

If an allelopathic interaction is to occur between plant A (sender) and plant B (receiver): (1) plant A must produce an active substance; (2) the substance must leave plant A; and (3) the substance must reach plant B in a concentration sufficient to influence its growth (Newman, 1978). Without question, MeJA is a highly active compound. Sagebrush produces MeJA in prodigious quantities (Farmer and Ryan, 1990; Karban et al., 2000; Hildebrand et al., 2000; Preston et al., 2001) and releases it into its surrounding environment by both volatile and aqueous transport (Karban et al., 2000; Preston et al., 2001). Here, we demonstrate that MeJA is present in sufficient quantities to affect the seed bank of *N. attenuata*.

If MeJA is an active component released by sagebrush that inhibits *N. attenuata* germination, it must enter the environment in amounts that would be active even after environmental dilution. Sagebrush releases MeJA by both volatilization and leaching. The volatile release of MeJA by undamaged sagebrush in the field is approximately 10–20 ng/g/hr (Karban et al., 2000). An average sagebrush plant may have a leaf mass of approximately 1 kg, suggesting that MeJA is released from a single plant at 10–20 μg/hr. Run-off collected by spraying sagebrush with water has approximately 2 ng MeJA/ml (Preston et al., 2001), and water extracts of sagebrush litter contained approximately 200 ng MeJA/ml. Moreover, we estimated the amount of MeJA that accumulates in the A_1 soil layer by approximating the MeJA content of the soil (1:1, potting soil–sand) collected from sagebrush plants growing in the growth chamber. To determine the recovery rate of MeJA, soil not exposed to sagebrush plants was treated with a known amount of MeJA. Samples were placed in volatile trapping containers and positioned in a water bath at 50°C. The MeJA volatilized from the soils was trapped as described in Methods and Materials (see *Volatile Exposure to MeJA and Sagebrush Affecting Germination of* N. attenuata). The average recovery rate from the soil with the known quantity of MeJA was determined to be approximately $8.46 \pm 1.50\%$. Using this recovery rate and assuming a linear relationship, we estimated the MeJA content in soils to be 11 and 14 ng/g under young and mature sagebrush plants, respectively. Germination of *N. attenuata* was significantly inhibited by 1.12 μg MeJA/seed cup (56 ng/seed; Figure 3). When considering the multiple routes of

transport, it is reasonable that MeJA is present in sufficient quantities to influence the seed bank of *N. attenuata*.

One of the principal criticisms of research on allelopathy is the frequent lack of correlation between laboratory bioassays and field studies or observations (Wardle et al., 1996). In the field, germination of *N. attenuata* is inhibited by an unidentified component located under and around sagebrush (Figure 1), with lower germination on soils collected from directly underneath sagebrush than on soils collected from between sagebrush plants and outside of the sagebrush population (Figure 2). This interaction seems to be controlled by adult sagebrush, as *A. tridentata* seeds and seedlings did not affect the germination response of *N. attentuata* seeds when sharing the small environment of a seed cup. This inhibition of germination can be simulated by exposure to volatile emissions of sagebrush and MeJA (Figure 5) and applications of water extracts of sagebrush litter and aqueous solutions of MeJA (Figure 7 and Figures 3 and 4, respectively). By manipulating MeJA levels in natural sagebrush products [chaff (Figure 6), water extracts (Figure 7)], we found a strong negative correlation between *N. attenuata*'s germination and the amounts of MeJA present. However, there is a discrepancy between the MeJA amounts that inhibited germination in the aqueous-and sagebrush water extract-based bioassays. The 200 ng MeJA/ml that was contained in the MeJA-supplemented sagebrush extract dilutions was 11.2 times less than the amount of MeJA that inhibited germination in aqueous dilutions. This may be explained either by the lipophilic components in the sagebrush litter water extract helping to disperse the otherwise insoluble MeJA and increasing its delivery to the seeds or possibly by a synergism occuring between the MeJA and the negative germination signal. In contrast to *N. attenuata*, seeds of *A. tridentata* are significantly less sensitive to applications of MeJA, requiring 300 times the amount of MeJA to inhibit germination. This is consistent with a fundamental prediction about the release of allelochemicals. If a plant is to release an active compound as a general and effective toxin into its surrounding environment, it must become desensitized to the compound itself. Moreover, four of five other *Nicotiana* species that are not known to occur with sagebrush required either the same or more MeJA than sagebrush to inhibit germination. This suggests that *N. attenuata* and *N. trigonophylla* may have evolved sensitivity to their MeJA-releasing neighbor, preventing them from germinating in disadvantageous competitive situations, and the idea further supports that MeJA is an active allelopathic compound in this habitat. Therefore, our bioassays in the laboratory reflect our results from the field. Moreover, we identified the active chemical component.

The final step in demonstrating an allelopathic interaction and the active chemical component responsible for mediating the interaction is the application of the proposed chemical under natural conditions, following the criteria set-up by Koch's postulates (Harper, 1977). While Koch's postulates, proposed for studying disease, are difficult to apply to allelopathic research due to fundamental

differences between how disease spreads in a body (by replication and one-to-one correspondence for microbe and disease) and the activity of allelopathic compounds in the environment [no replication and, instead, degradation as well as possible syngerisms between different components that do not produce one-to-one relationships between chemicals and allelopathic response (Williamson, 1990)], they provide a framework within which to test proposed allelopathic substances. The ultimate test will likely come through genetic manipulation of the plants with proposed allelopathic tendencies to eliminate the release of the putative active components, placing them back into the natural environment and observing the effects. In such an experiment, all other variables, such as resource competition, shading, releases of other chemicals, etc., remain unchanged while the putative toxin is absent. In the absence of a sagebrush plant that does not emit MeJA, our combinations of field and laboratory experiments, nontheless, provide strong evidence that MeJA mediates interactions between sagebrush and seeds of *N. attenuata*.

This example of allelopathy should not necessarily be considered a negative interaction between these species. Inhibition of *N. attenuata* germination likely benefits both the sagebrush plant (by reducing competition) and the *N. attenuata* seed bank (by preserving the seed bank of a poor competitor until competition levels are reduced, most likely by fire). Eavesdropping on chemical signals in its surrounding environment appears to be crucial for *N. attenuata* to locate its postfire niche (Preston and Baldwin, 1999). It may be common that species specializing in this niche are sensitive to the release of substances by later successional species. Annuals also showed reduced density numbers near *Encelia farinosa* (Compositae), including *Emmenanthe penduliflora* (Went, 1949; Friedman, 1987), another annual that germinates in response to component(s) in smoke (Keeley et al., 1985; Keeley and Pizzorno, 1986). However, since MeJA is such a biologically active substance for a large number of plants, it should be considered further in other studies on plant–plant interactions in *Artemisia*-dominated landscapes.

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ODORANT BINDING PROTEIN HOMOLOGUES OF THE MALARIA MOSQUITO *Anopheles gambiae*; POSSIBLE ORTHOLOGUES OF THE OS-E AND OS-F OBPS OF *Drosophila melanogaster*

RICHARD G. VOGT[1,*]

[1]*Department of Biological Sciences*
University of South Carolina
Columbia South Carolina 29208

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Abstract—Twenty-nine *Anopheles gambiae* candidate Odorant Binding Proteins (OBPs) were characterized for similarity to OBPs of *Drosophila melanogaster* and other insects. Twenty-five of these sequences were identified by BLAST searching the *A. gambiae* genome database. Several *A. gambiae* sequences were significantly similar to the *D. melanogaster* OBPs OS-E/OS-F, LUSH and PBPRP2/PBPRP5. Exon boundary comparisons suggests that two *A. gambiae* genes are orthologues of OS-E and OS-F, justifying the names *Agam*OS-E (EAA01090, AF437886) and *Agam*OS-F (EAA14641, AF437884). If these are orthologues, then the gene duplication establishing the OS-E and OS-F lineages predated the divergence of mosquitoes and flies. The identification of orthologous OBPs and other chemosensory genes between *D. melanogaster* and *A. gambiae* should accelerate the transfer of physiological and behavioral information between these two species.

Key Words—Odorant Binding Protein, OBP, evolution, odor detection, pheromone detection, chemosensory proteins, *Anopheles gambiae*, mosquito, olfaction.

INTRODUCTION

Odorant Binding Proteins (OBPs) are small water-soluble proteins thought to transport odors to receptor proteins in olfactory neuron membranes (Vogt et al., 1999). OBPs comprise a multigene family; thirteen have been identified in *Manduca sexta* through direct sequencing, and 38 in *Drosophila melanogaster* through direct sequencing and genome analysis (Robertson et al., 1999; Galindo and Smith, 2001;

* To whom correspondence should be addressed. E-mail: VOGT@BIOL.SC.EDU

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Graham and Davies, 2002; Vogt et al., 2002). OBPs are differentially expressed among olfactory sensilla, contributing to the unique phenotypes and odor sensitivities of these sensilla (de Bruyne et al., 2001; Shanbhag et al., 2001). In this study, OBP genes of *D. melanogaster* and other insect species were used to identify OBPs from the *A. gambiae* genome, and the likely *A. gambiae* orthologues of the *D. melanogaster* OBPs OS-E and OS-F (also known as PBPRP3) are identified.

METHODS

Amino acid sequences of previously reported OBPs were used in a BLAST-P search of the *A. gambiae* genome through the NCBI web site (8/2002). This database identifies hypothetical gene coding regions and the corresponding amino acid sequences. Sequences with an E-value of 0.05 or lower were accepted. Sequences were aligned using ClustalX, and a neighbor joining tree was constructed using MEGA2. No effort was made to modify sequences, although previously reported versions of these sequences were used if available. Exon boundaries were determined by translating the genomic sequences into multiple reading frames to identify the amino acid sequences bounding exon/intron transitions.

RESULTS AND DISCUSSION

Table 1 lists 29 candidate OBP genes from *A. gambiae.* Twenty-five unique genes were identified through BLAST search analysis of the *A. gambiae* genome and are listed with the protein accession number (EAA#) and the corresponding gene scaffold (AAA#). Four pairs of identified sequences were considered to represent the same genes; small variations in nucleic acid and amino acid sequences and very low variation in third base positions of codons suggested that differences between members of each pair was either due to allelic variation or sequencing error. Six of the 25 sequences were previously reported from direct sequence analysis (Biessmann et al., 2002). Three additional sequences were also previously reported as *A. gambiae* OBP. The *Agam*D7 gene, encoding a salivery protein, was also identified as a candidate OBP homologue.

Figure 1A shows a sequence similarity tree assembled from an alignment of the 29 candidate *A. gambiae* OBP sequences, 38 *D. melanogaster* OBP sequencs (Galindo and Smith, 2001; Graham and Davies, 2002; Vogt et al., 2002) and OBP related sequences from diverse other species. The long-branch lengths in this tree indicate the considerable sequence divergence between most of these sequences. Most sequences are in branches specific to a given insect group, though several similarity groups do include multiple insect groups. Three similarity groups include both A. gambiae and D. melanogaster sequences: OS-E/OS-F, LUSH, and PBPRP2/PBPRP5. The OS-E/OS-F group includes 1 sequence from Culex

TABLE 1. ACCESSION NUMBERS OF *A. Gambiae* OBP CANDIDATES

1	EAA09615 (AAAB01008904) (*Agam*OS-F) EAA14641 (AAAB01008982) AF437884 (OBP-1 Biessmann et al., 2002)	2	EAA03140 (AAAB01007923) EAA08879 (AAAB01008888) AF437889 (OBP-6 Biessmann et al., 2002)
3	EAA01090 (AAAB01008987) (*Agam*OS-E) AF437886 (OBP-3 Biessmann et al., 2002)	4	EAA09958 (AAAB01008933) AF437887 (OBP-4 Biessmann et al., 2002)
5	EAA14656 (AAAB01008982) AF437885 (OPB-2 Biessmann et al., 2002)	6	EAA01289 (AAAB01008987) AF437890 (OBP-7 Biessmann et al., 2002)
7	EAA00498 (AAAB01008986) EAA03297 (AAAB01008194)	8	EAA03447 (AAAB01008582) EAA09285 (AAAB01008898)
9	EAA00779 (AAAB01008986)	10	EAA06803 (AAAB01008847)
11	EAA00788 (AAAB01008986)	12	EAA07741 (AAAB01008859)
13	EAA00801 (AAAB01008986)	14	EAA07997 (AAAB01008859)
15	EAA01052 (AAAB01008987)	16	EAA09324 (AAAB01008900)
17	EAA01392 (AAAB01008987)	18	EAA10932 (AAAB01008960)
19	EAA10491 (AAAB01008987)	20	EAA12996 (AAAB01008966)
21	EAA03742 (AAAB01008799)	22	EAA13515 (AAAB01008976)
23	EAA03745 (AAAB01008799)	24	EAA14622 (AAAB01008982)
25	EAA06799 (AAAB01008847)		
26	AF393485 (OBPI, Robertson et al., Submission 2002)	27	AF437888 (OBP-5 Biessmann et al., 2002)
28	AF393487 (OBP2, Robertson et al., Submission 2002)	29	AF457552 (D7 Salivary Protein, Francischetti et al., Submission 2002)

quinquefasciatus (Ishida et al., 2002) and 4 sequences from A. gambiae; this similarity group was further characterized.

OS-E and OS-F are the most similar of the *D. melanogaster* OBPs (Vogt et al., 2002). The OS-E/OS-F genes are adjacent within a 5000 nucleotide region of chromosome 3, have conserved exon/intron boundaries, and are co-expressed in a subset of olfactory sensilla in the adult antenna, and are, thus, assumed to be co-derived from a gene duplication event (Hekmat-Scafe et al., 2000; Vogt et al., 2002).

Figure 1B represents an amino acid alignment of the 7 proteins of the OS-E/OS-F group. All 7 proteins contain 6 cysteins that co-align. Exon boundaries were determined for each sequence (the *Cluex* gene was not available). *Dmel*OS-E and *Dmel*OS-F have two conserved exon boundaries at positions 79 and 104. *Dmel*OS-F has an additional short exon at the extreme C-terminal end (position 194) encoding 5 amino acids (HYFLP), which align with a similar sequence in *Dmel*OS-E (HYFLV). *Agam*(EAA01090) also contains exon boundaries at alignment positions 79 and 104 as well as the C-terminal sequence HYFIP, but lacks a C-terminal exon. *Agam*(EAAl4641) has an exon boundary at alignment position 104 and a C-terminal exon at position 194 with tbe sequence HYFLV, but lacks the position 79 boundary. *Agam*(EAAl4656) and *Agam*(EAAl4622) have only single exons. The genes of *Agam*(EAAl4656), *Agam*(EAAl4622), and *Agam*(EAAl4641) reside in a cluster; *Agam*(EAA14656) and *Agam*(EAAl4622) are separated from

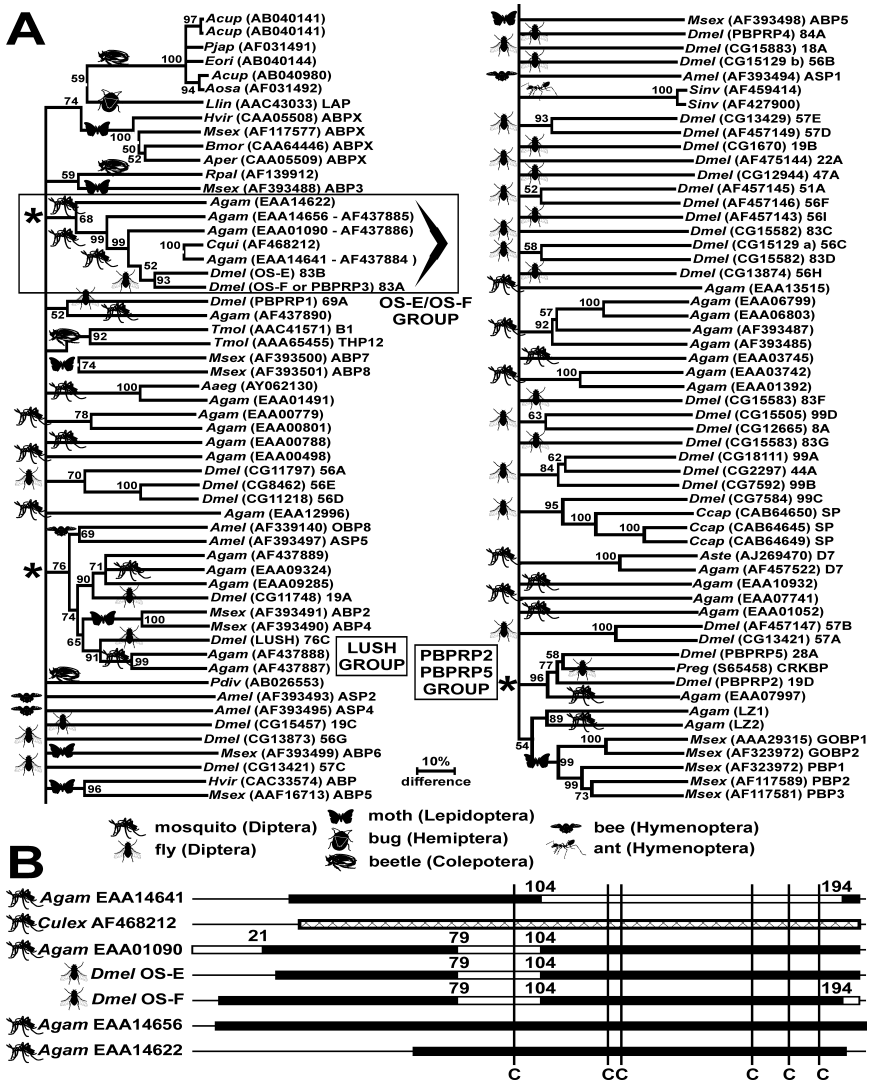


FIG. 1. A. A neighbor joining tree is shown; branches are collapsed to 50% bootstrap support based on 1000 replicates. The single tree was separated in two for ease of viewing. *Agam*LZ1 and *Agam*LZ2 are unpublished from Larry Zwiebel. B. A graphical representation of an amino acid alignment of proteins belonging to OS-E/OS-F similarity group (1A). The overall lengths represented include alignment gaps; the numbers refer to the amino acid position at the C-terminal side of a domain in the aligned sequence. Exon domains are indicated by alternating white-filled and black-filled; vertical lines indicate positions of conserved cysteins (C).

each other by about 3,000 base pairs (bp) and from *Agam*(EAAl4641) by about 200,000 bp. *Agam*(EAA01090) is separated from this cluster by about 40 million bp.

The conservation of exon boundaries suggests the evolutionary history of these genes. The intron-containing *Agam*(EAAl4641) and *Agam*(EAA01090) are presumably derived from a gene duplication based on sequence similarity and conservation of exon boundaries; the two genes presumably later became separated by a translocation event. The gene cluster of *Agam*(EAAl4641), *Agam*(EAAl4656), and *Agam*(EAAl4622) likely derived through gene duplications, since tbe probability of related genes becoming located near each other by translocation is small given tbe overall size of the genome. If this cluster did derive through gene duplication, then *Agam*(EAAl4641) is the likely founder since its relation to *Dmel*OS-E and *Dmel*OS-F suggests an ancestry that predates the divergence of flies and mosquitoes. It is not obvious how *Agam*(EAAl4641) could have produced an intron free copy that then duplicated to *Agam*(EAAl4656) and *Agam*(EAAl4622); perhaps the *Agam*(EAAl4641) transcript was captured by a retrovirus and reinserted into the genome with some homologous recombinatorial bias. This gene cluster is absent in *D. melanogaster* (Vogt et al., 2002) and, thus, may have arisen independently in mosquitos.

Agam(EAA01090) and *Agam*(EAAl4641) are probable orthologues of *Dmel*OS-E and *Dmel*OS-F. Hekmat-Scafe et al. (2000) characterized these genes from multiple *Drosophila* species and suggested OS-E/OS-F diverged early in the evolutionary history of flies. *Agam*(EAA01090) contains both the 79 and 104 exon boundaries, but lacks the 194 boundary that characterizes *Dmel*OS-F. *Agam*(EAAl4641) contains the position 194 exon boundary of *Dmel*OS-F but lacks the position 79 boundary characteristic of both *Dmel*OS-E and *Dmel*OS-F. Assuming that loss of an existing intron is more probable than the random gain of an intron at a specific position, then the presence of the position 194 boundary suggests that *Agam*(EAA01090) and *Dmel*OS-E are orthologues, and *Agam*(EAA01090) might be named *Agam*OS-E. Similarly, *Agam*(EAAl4641) and *Dmel*OS-F are apparent orthologues, but *Agam*(EAAl4641) has independently lost one of its introns (position 79); *Agam*(EAAl4641) might be named *Agam*OS-F. The duplication that established these two gene lineages apparently occurred before the divergence of mosquitoes and flies.

Not all members of the OBP gene family are involved in olfaction (Vogt et al., 2002), and no dipteran OBPs including OS-E and OS-F have actually been shown to bind odor molecules. However, the demonstrated expression of OS-E and OS-F in olfactory sensilla of *Drosophila* (Hekmat-Scafe et al., 1997) and the structural similarities of these two proteins to OBPs that have been shown to bind odors supports their role in odor detection. The conservation of these genes between mosquito and fly suggests OS-E and OS-F have a singularly important function in both species groups.

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EFFECT OF NITROGEN AND WATER TREATMENT ON LEAF CHEMISTRY IN HORSENETTLE (*Solanum carolinense*), AND RELATIONSHIP TO HERBIVORY BY FLEA BEETLES (*Epitrix* SPP.) AND TOBACCO HORNWORM (*Manduca sexta*)

MARTIN L. CIPOLLINI,[1,*] ERIC PAULK,[1] and DONALD F. CIPOLLINI[2]

[1]*Department of Biology*
430 Berry College
Mount Berry, Georgia 30149, USA

[2]*Department of Biological Sciences*
Wright State University
3640 Colonel Glenn Highway
Dayton, Ohio 45435-0001, USA

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Abstract—We studied the interaction between plants (horsenettle; *Solanum carolinense*) and herbivorous insects (flea beetles; *Epitrix* spp., and tobacco hornworm; *Manduca sexta*) by focusing on three questions: (1) Does variation in nitrogen availability affect leaf chemistry as predicted by the carbon-nutrient balance (CNB) hypothesis? (2) Does variation in plant treatment and leaf chemistry affect insect feeding? (3) Is there an interaction between the insect herbivores that is mediated by variation in leaf chemistry? For three successive years (1998–2001), we grew a set of clones of 10 maternal plants under two nitrogen treatments and two water treatments. For each plant in the summer of 2000, we assayed herbivory by hornworms in both indoor (detached leaf) and outdoor (attached leaf) assays, as well as ambient flea beetle damage. Estimates of leaf material consumed were made via analysis of digitized leaf images. We also assayed leaves for total protein, phenolic, and glycoalkaloid content, and for trypsin inhibitor, polyphenol oxidase, and peroxidase activity. Despite strong effects of nitrogen treatment on growth and reproduction, only total protein responded as predicted by CNB. Leaf phenolic levels were increased by nitrogen treatment, polyphenol oxidase activity was decreased, and other leaf parameters were unaffected. Neither hornworm nor flea beetle herbivory could be related to plant treatment or genotype or to variation in any of the six leaf chemical parameters. A negative relationship between flea beetle and hornworm herbivory was found, but was not apparently mediated by any of the measured leaf chemicals. Because leaf resistance was maintained in low nitrogen plants at the apparent

* To whom correspondence should be addressed. E-mail: mcipollini@berry.edu

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expense of growth and reproduction, our results support the concept of a fitness cost of defense, as predicted by the optimal defense hypothesis.

Key Words—Carbon–nutrient balance, cost of defense, *Epitrix*, induced defense, insect herbivory, *Manduca sexta*, nitrogen supplementation, optimal defense, plant secondary metabolites, *Solanum carolinense*.

INTRODUCTION

It is well known that plants can physiologically respond to environmental factors (Tuomi et al., 1984; Waterman and Mole, 1989; Baldwin, 1994) and that these changes can affect the actions of organisms with which they interact (Stamp, 1992; Baldwin 1999). For instance, if the secondary metabolite content of the foliage changes in response to some environmental factor, rates of foliar herbivory may also change (Karban and Baldwin, 1997). In this paper, we address two interrelated issues that concern changes in foliar chemistry over time: (1) plant responses to variation in available nutrients, and (2) chemical responses induced by herbivory. The first issue has attracted much attention in recent years, and several hypotheses have been constructed to explain patterns of defense production under different nutrient regimes. In the carbon–nutrient balance (CNB) hypothesis (Bryant et al., 1983), plants should allocate relatively more carbon resources to nitrogen-containing secondary metabolites when the plant has an abundance of nitrogen at its disposal (Muzika, 1993; Gebauer et al., 1998; Mutikainen et al., 2000). Conversely, the CNB hypothesis predicts decreases in strictly carbon-based compounds under conditions of increased nitrogen availability. Other major hypotheses concerning plant response to environmental variation include the growth–differentiation balance (GDB) (Hermes and Mattson, 1992), resource availability (RA) hypothesis (Coley et al., 1985; Bryant et al., 1985), and optimal defense (OD) hypothesis (Rhoades, 1979). Both the CNB and GDB hypotheses may be considered "supply-side" hypotheses, in that they assume that the supply of substrates is the primary controlling factor influencing quantitative variation in secondary metabolites. Conversely, the RA and OD hypotheses may be considered adaptive, "demand-side" hypotheses, which assume that demands placed upon tissues (e.g., for defense) and the selective value of that tissue to the plant are the primary factors influencing quantitative variation in secondary metabolites. While criticism of the CNB hypothesis has been on the rise in recent years (e.g., Gershenzon, 1994; Hamilton et al., 2001), its predictions remain a common framework around which studies of the influence of resource variation upon secondary metabolite variation are built.

The second major issue (induced defenses) has also attracted much attention in recent years. Levels of plant secondary metabolites and defense proteins can change in response to leaf damage (e.g., from insect feeding), and such changes can affect

subsequent feeding by herbivores or plant resistance to microbes and other parasites (e.g., Haukioja, 1991; Baldwin, 1994; Karban and Baldwin, 1997). Under certain circumstances, such effects may result in negative interactions among different herbivore species. This may be particularly true when feeding by one type of herbivore follows feeding by another. Some studies (e.g., Baldwin et al., 1993; Stout et al., 1998; Bolter et al., 1998; Cipollini and Bergelson, 2001) have shown that variation in nutrient availability can affect the induction of chemical responses in plants. However, the extent to which such chemical responses have consequent effects upon herbivory has been little explored (cf., Hartley and Jones, 1997). This area is a critical link between hypotheses concerning plant responses to nutrient variation and those concerning induced responses to herbivory.

We addressed these issues via a study of the interactions between two specialist insect species, *Epitrix* spp. (flea beetles) and *Manduca sexta* (hornworm), and the perennial plant *Solanum carolinense* L. (horsenettle). The plants used in this study were included in an on-going study examining the effect of added nitrogen on plant growth, reproduction, and fruit chemistry for three growing seasons (from the spring of 1998 through the summer of 2000; see details in Methods and Materials). Nitrogen addition approximately doubled plant size in all three years (M. Cipollini et al., unpublished data), which demonstrated the consistent and strong growth response to nutrient variation that must be obtained when addressing the CNB hypotheses. In all three years, variation in plant size produced (by allometric effects) increased fruit number, suggesting strong direct effects of nutrient variation on individual plant fitness. Despite large effects on growth and reproduction, levels of leaf trypsin inhibitor activity (TI), total glycoalkaloid (TGA), total protein, and total phenolics in 1999 were influenced by N addition in ways inconsistent with CNB hypothesis (M. Cipollini et al., unpublished data). In particular, leaf TI and TGA (nitrogen-containing metabolites) levels were unaffected, and total phenolics (carbon-based metabolites) were increased in response to nitrogen addition. Furthermore, none of the leaf chemical variables were found to relate to ambient leaf damage by flea beetles. These results were considered tentative because several potentially important parameters were not measured [e.g., leaf peroxidase (POD) and leaf polyphenol oxidase (PPO)]. Additionally, our simple method of measuring flea beetle damage (counting holes on leaves) may have been inaccurate.

As a follow-up to these earlier results, we initiated a study in the summer of 2000 using the same plants and general study design, but focusing more explicitly on the effects of plant treatment on leaf chemistry and herbivory. Our goal was to assess leaf chemistry more fully and to assess insect damage more accurately via image analysis of photographed leaves. Additionally, we sought to quantify hornworm feeding and growth rate through the use of both indoor and outdoor feeding trials, with the goal of determining if these parameters were influenced either by leaf chemistry and/or by ambient levels of flea beetle damage.

We focused on the following specific questions in our 2000 study: (1) Does plant treatment affect leaf chemistry in a manner consistent with the predictions of CNB theory? (2) Regardless of the effect of plant treatment, does variation in leaf chemistry affect feeding by the two herbivores? (3) Is there evidence of an interaction between flea beetle and hornworm herbivory that may be mediated by induced changes in leaf chemistry?

Based upon CNB theory, we predicted that nitrogen treatment should elevate levels of nitrogen-containing metabolites (total protein, TI, PPO, POD, and TGA) and decrease levels of strictly carbon-based metabolites (phenolics). Because we observed large effects of nitrogen treatment on plant size and reproduction, we expected to see large effects on leaf chemistry and that these changes should affect herbivory. Total protein was expected to positively influence herbivory, whereas the other leaf chemical parameters were expected to reduce herbivory; thus, no *a priori* prediction was made concerning the net effect of the any chemical changes. Based on our observations of changes in leaf morphology following flea beetle attack (e.g., browning and apparent lignification around feeding holes), we expected changes induced by flea beetles to affect subsequent feeding by hornworms. Thus, based upon induced defense theory, we expected answers to our second and third questions to be affirmative. Finally, we asked the more general question of whether variation in nutrient availability might affect any induced responses (thus linking our first question to the second two).

METHODS AND MATERIALS

Experimental Planting Design. In the fall and winter of 1997–1998, 12 clones from each of 10 different maternal plants (Table 1) were propagated from root

TABLE 1. SOURCES OF ORIGINAL *S. carolinense* MATERNAL PLANTS[a]

Code	Date	Collection location (USA)
AUG	1993	Augusta, Georgia
EAST	1991	Easton, Maryland
HOW	1991	Howard County, Maryland
KENT	1991	Kent Island, Maryland
N2O	1991	Smithsonian Environmental Research Center, Edgewater, Maryland
SACC	1991	Conservation and Research Center, Front Royal, Virginia
SEN	1991	Seneca Rocks, West Virginia
SERC	1991	Smithsonian Environmental Research Center, Edgewater, Maryland
TIFT	1993	Tifton County, Georgia
TWIN	1991	Kent Island, Maryland

[a] Collections of root-stock from a single plant were made at sites no closer than several km apart.

cuttings under greenhouse conditions. Plants were collected from throughout the natural range of *S. carolinense*, from sites separated by several miles to hundreds of miles, with the idea of maximizing potential genetic variation among the clones. Nevertheless, clones derived from near one another might be expected to be more genetically similar, and, thus, might show similar responses. In early April 1998, each clone was transplanted into a 12-liter pot filled with a sandy loam–clay soil mixture made to mimic a typical southeastern US soil. The 120 clones were arranged in a fully randomized array with pots 1 m apart, surrounded by a 2.5-m-high wire mesh fence designed to deter mammalian herbivores but to allow access by pollinators and other insects. Three plants from each clone were subjected to the following manipulations: (a) control (C): to minimize drought stress, watered only if any of the plants in the entire array appeared wilted (when this was done, all 120 plants were watered); (b) water addition (W): watered to complete soil saturation three additional times per week; (c) nitrogen addition (N): 10 g dry ammonium nitrate fertilizer added to the pot four times during each year (mid-April, -May, -June, and -July); and (d) water and nitrogen (N + W): addition of both water and nitrogen. Pots were randomly rearranged on an annual basis, and treatments were continued on the same set of plants until the fall of 2000.

Outdoor Hornworm Feeding Trials and Flea Beetle Damage Assessment. To quantify levels of herbivory by hornworms, we purchased a lab strain from Carolina Biological Supply. A total of about 500 larvae were raised from eggs within individual glass vials on a generic meal provided with the eggs. Incubation of larvae was at 28°C, 14 hr light/10 hr dark, and 50–70% relative humidity. Starting June 20, 2000, terminal branches from each of the 120 plants were selected and photographed in color with a digital camera (Nikon Coolpix 950; 300 dpi) by placing the leaves between a gridded background (to allow subsequent size scaling of images) and a clear Plexiglas plate. The branch was then placed in a 600-ml plastic foam cup along with a single third- to fifth-instar larvae at about 17:00 hr. Lids were fitted tightly to prevent escape of the larvae. Each larva was allowed to feed overnight (15–17 hr). After the feeding trial had ended, the branch was photographed again, larvae were weighed, and the digital photographs were downloaded onto a computer as color jpeg images. This process was completed for all plants over the course of five days. Immediately following each trial, 15 leaves were randomly collected from each plant, freeze-dried, and ground to pass a #40 mesh screen. Another five leaves were randomly collected for use in assessing flea beetle damage. Each of these five leaves was photographed against a backlit screen using the digital camera. These images were downloaded onto a computer as color jpeg images.

Quantification of Growth and Reproductive Variables. Plants were allowed to continue to grow, set fruit, and senesce naturally in the fall. At the end of the fruit-ripening season (late November), all ripe fruits were collected, counted, and

weighed, and all above-ground stem mass was collected, dried to constant mass, and weighed.

Image Conversion and Filtering. All images were filtered using the "threshold" function of Graphic Workshop for Windows (Alchemy Mindworks, Inc., 1997) to produce monochrome bitmaps. Threshold values (80–130) were used to ensure that leaf material converted to black, while missing leaf material converted to white. Bitmap files were size-standardized and cleaned up using Microsoft Paint for Windows (Microsoft, Inc., 1991–1998).

Image Analysis of Hornworm Feeding. Using the "histogram" function of the Image J program (National Institute of Health, 2000), the number of black pixels (corresponding to leaf area) was determined for each image and for an image of a black square of known area (49 cm^2). Pixel counts were converted to leaf wet mass based upon mass per unit area determinations on two leaves from each plant. We calculated total consumption from estimates of leaf mass at the beginning and end of each feeding trial, and then divided total consumption by larval final mass to calculate relative consumption rate (RCR).

Image Analysis of Flea Beetle Feeding. Using the "histogram" function of the Image J program, we quantified the number of black (leaf material) and white (missing leaf material) pixels for each leaf image. We divided the number of white pixels by the total pixel count to estimate flea beetle damage as proportion of leaf area consumed. Nearly all leaf damage could be ascribed to flea beetles, based upon the uniformly small round holes typically produced by flea beetle species (Mauricio, 1998), and the observation that few other types of herbivorous insects attacked leaves of our study plants.

Indoor Hornworm Feeding Trials. At the same time as outdoor hornworm trials were taking place, we confined individual preweighed second to fourth instars with individual preweighed detached leaves in Petri dishes for 18-hr feeding trials. Petri dishes were maintained under conditions identical to those used for rearing the larvae. For each of four successive trials, we used a single leaf from each of the 120 plants. For each trial, we also placed 20 randomly-selected leaves individually in Petri dishes without larvae for the same period of time, to allow for calculation of expected mass change due to water loss alone. At the end of each trial, we reweighed all leaves and larvae. To determine mass consumed per trial, we estimated the mass of each leaf in absence of herbivory using regression equations based upon isolated leaves (R^2 ranging from 0.952 to 0.989). Actual leaf mass was subtracted from the estimated leaf mass to calculate mass consumed per trial. Leaf mass consumed was divided by the initial larval mass to calculate relative consumption rate (RCR). Mass gained within each trial was divided by the initial larval mass to calculate relative growth rate (RGR) of the larvae. Leaf mass consumption was summed across trials as a means of estimating total consumption, and total mass gain and total relative growth across all four trials was determined using initial and final larval masses.

Leaf Chemical Variables

Leaf Peroxidase (POD). Peroxidase is an enzyme that catalyzes the polymerization of phenolic alcohol monomers into lignin. In addition to its role in lignin formation, POD has roles in the formation of phenoxy and oxygen radicals and in the regulation of auxin levels (Campa, 1991). Because POD contains nitrogen, we predicted that increased nitrogen availability would result in increased POD levels. Mechanical damage, insect wounding, pathogen attack, and addition of wound signaling hormones like salicylic acid and jasmonic acid have been shown to increase POD activity, and increased POD activity has been associated with increased resistance to herbivores (Thaler et al., 1996; Cipollini and Redman, 1999). To assay POD activity, soluble proteins were extracted from freeze-dried and ground leaf samples by incubating 10 mg of sample in 1.5 ml 0.05 M sodium phosphate buffer (pH 6.8) containing 7% PVPP, 1.67 mM phenylthiourea, 0.3 M KCl, and 0.4 mM ascorbic acid. POD activity in soluble protein extracts was determined by following the oxidation of guaiacol in the presence of hydrogen peroxide at 470 nm, as in Cipollini (1998), in an assay adapted for a microplate reader. POD activity was expressed as a change in absorbance at 470 nm per minute per milligram extract protein. Soluble protein content was determined in leaf extracts using the Bradford assay (Bradford, 1976), wth Bio-Rad protein dye reagent and bovine serum albumin as a standard.

Leaf Trypsin Inhibitors (TI). Trypsin inhibitors are antinutritive proteins that competitively inhibit digestive serine proteases of animals and other organisms (Koiwa et al., 1997). As with POD, these proteins were predicted to increase in response to increased nitrogen availability. Trypsin inhibitors have been shown to be inducible in several plant species, and to be affected by nutrient availability (Stout et al., 1998; Cipollini and Bergleson, 2000, 2001). However, flea beetles, like all coleopterans, rely on cysteine proteases for digestion of leaf proteins rather than serine proteases, so it is unlikely that flea beetles would be affected by constitutive or induced variation in TI. However, flea beetle damage may induce increases in TI, to which hornworms should be sensitive. The TI content was determined by examining the radial diffusion of soluble protein extracts through a trypsin-containing agar as in Cipollini and Bergelson (2000). TI content of extracts was expressed as micrograms of TI per milligram extract protein.

Leaf Polyphenol Oxidase (PPO). Polyphenol oxidases are enzymes that catalyze the oxidation of phenolic compounds. Such oxidation can result in quinone formation, polymerization, and other changes that can greatly affect the biological activity of phenolics present in plant tissues (Appel, 1993). As with POD and TI, PPO levels were predicted to increase in response to increased nitrogen availability. It has been demonstrated that PPOs are inducible and associated with increased insect resistance in tomato (Thaler et al., 1996; Cipollini and Redman, 1999). We

assayed leaf protein extracts for PPO activity by following the oxidation of caffeic acid to its colored quinone complex (Thaler et al., 1996) in an assay adapted for a microplate reader. PPO activity was expressed as a change in absorbance at 470 nm per minute per milligram extract protein.

Leaf Total Protein (PROT). As for individual enzymes, total protein was expected to increase in response to increased nitrogen availability; however, no specific prediction was made concerning the effect of leaf damage on total protein content as all manner of responses have been reported in the literature (Baldwin, 1994). Numerous studies have shown that insect feeding may be affected by elevated protein and that the relative growth rates of insects may be enhanced (e.g., Hare, 1987). We analyzed freeze-dried leaf samples for total dietary protein using the Bradford (1976) assay as modified for plant material by Jones et al. (1989), using ribulose 1,5-biphosphate (the most prevalent protein in plant leaves) as an external standard.

Leaf Total Phenolics (PHEN). Phenolics, a group of miscellaneous aromatic compounds ultimately derived from cinnamic acid, may negatively influence feeding and relative growth rates of herbivorous insects through a variety of mechanisms. Many individual compounds and "total phenolics" have been shown to be inducible (Baldwin, 1994; Dixon and Paiva, 1995), and the CNB hypothesis suggests that, since they are carbon-based secondary metabolites, phenolics will decrease in response to increased nitrogen availability (Baldwin, 1994). We analyzed freeze-dried leaf samples for total phenolics using the Coomassie brilliant blue technique (Budini et al., 1980) with the modifications of Graham (1992) and using chlorogenic acid (the most prevalent phenolic in horsenettle leaves) as an external standard.

Leaf Total Glycoalkaloids (TGA). Glycoalkaloids are nitrogen-containing steroidal bases, active against a variety of organisms ranging from birds and mammals to insects and fungi (Hare, 1983; Tingey, 1984; van Gelder, 1990; Cipollini and Levey, 1997a,b). Because glycoalkaloids contain nitrogen, we expected that leaf TGA should be elevated in leaves of nitrogen treated plants, although reports have suggested otherwise for this group of compounds (Nowacki et al., 1976; Hoffland et al., 1999). They have been shown to be inducible in a variety of studies, particularly those focusing on damage to potato tubers (e.g., van Gelder, 1990). We analyzed freeze-dried leaf samples for TGA with the method of Birner (1969) with modifications of van Gelder (1984) with solasodine (the sole N-containing steroidal base in horsenettle leaves) as an external standard.

Statistical Analyses

Outdoor Hornworm Trials (ANOVA/ANCOVA). The dependent variable RCR was analyzed using factorial analysis of variance (independent variables: treatment

and maternal plant), with and without leaf chemical variables as covariates. The incorporation of leaf chemical variables as covariates in this and subsequent analyses was designed to determine the degree to which differences among treatments and/or maternal plants were associated with variation in leaf chemistry.

Indoor Hornworm Trials (ANOVA/ANCOVA). The dependent variables RCR and RGR were analyzed using factorial repeated-measures analysis of variance (independent variables: treatment, maternal plant, trial), with and without leaf chemical variables as covariates. The dependent variables total consumption and total RGR were analyzed using factorial analysis of variance (independent variables: treatment and maternal plant), with and without leaf chemical variables as covariates.

Flea Beetle Damage. We analyzed the dependent variable proportion of leaf removed by flea beetles using factorial analysis of variance (independent variables: treatment and maternal plant), with and without leaf chemical variables as covariates.

Leaf Chemistry as a Function of Plant Treatment (MANOVA/ANOVA). Following transformation as necessary, the leaf chemical variables were included as dependent variables in a factorial multiple analysis of variance (MANOVA: independent variables: treatment and maternal plant). Because the MANOVA was significant, we analyzed each of the dependent variables separately using univariate factorial analysis of variance (ANOVA). These analyses were intended to determine which, if any, of the dependent variables were affected by our treatment conditions.

Correlations between Leaf Chemistry and Insect Feeding. We calculated Pearson correlations among all leaf chemical variables and insect feeding variables. The intent of these analyses was to determine if any of the variation in feeding or performance by the insects was associated with differences in leaf chemistry, regardless of effects of treatment and maternal plant. We adjusted P values for experiment-wise error rates using Bonferroni correction (Simple Interactive Statistical Analysis, 2001).

Effects on Fitness. Using total fruit wet mass as the dependent variable, we used ANCOVA to determine the effects of treatment, and maternal plant on plant fitness, with leaf chemical variables and flea beetle damage as covariates. Individual fruit mass correlated strongly with seed number in a sub-sample of our fruits ($N = 100$; $R = 0.84$; $P < 0.001$). This analysis was designed to determine relative contributions to each of the factors to our estimate of plant fitness.

RESULTS

Leaf Chemistry as a Function of Plant Treatment. Plants under high nitrogen treatments (N, N + W) showed increases in total proteins and phenolics and

a decrease in PPO (Table 2A). Our MANOVA analysis concerning the overall effects of plant treatment and maternal plant on leaf chemisty showed a strong overall effect of plant treatment on leaf chemistry, but no effect of maternal plant (Table 2B). These results could be attributed to significant increases in total protein and total phenolics and to a decrease in PPO in response to nitrogen addition, as supported by our MANCOVA results (Table 2C). While the increase in total protein in response to nitrogen treatment is in support of the CNB hypothesis, the positive effect of nitrogen addition on total phenolics, the lack of an effect on POD, TI, and TGA, and the negative effect on PPO were all in contrast to CNB-based predictions.

Outdoor Hornworm Trials. Based upon our estimate of relative consumption rate (RCR), hornworm feeding in outdoor trials was unrelated to plant treatment and did not vary significantly among maternal plants (clones) (Table 3A). Furthermore, none of the measured leaf chemical variables contributed significantly to the explanation of hornworm feeding, based upon our ANCOVA results (Table 3B).

Indoor Hornworm Trials. As for outdoor hornworm trials, larval total RCR was unaffected by plant treatment and maternal plant (Table 4A). In these trials, however, total phenolics showed a significant effect ($P < 0.05$) upon total RCR when incorporated into the ANCOVA analysis (Table 4B). Results for repeated-measures ANOVA mirrored the across-trial result in showing no effect of plant treatment or maternal plant on within-trial RCR (Table 4C), but a significant effect of total phenolics on RCR (Table 4D). Finally, repeated-measures results for within-trial RGR showed no effect of plant treatment or maternal plant (Table 4E), and no effect of any leaf chemical variable (Table 4F).

Flea Beetle Damage. Our assessment of flea beetle damage with respect to plant treatment, maternal plant, and leaf chemistry showed results generally similar to those found for hornworms (e.g., no effect of plant treatment or maternal plant on leaf damage; Table 5A), and no relationship with any of the leaf chemical variables (Table 5B). In the ANCOVA analysis (Table 5B), leaf POD showed a marginally significant relationship with leaf damage ($P = 0.055$), which may reflect a slight lignification response (or a response by the beetles to lignification).

Correlations between Leaf Chemistry and Insect Feeding. The only correlation that was significant was the negative correlation (Figure 1; $R = -0.276$) between flea beetle damage and feeding by hornworm larvae in outdoor trials (Table 6). In other words, none of the leaf chemical variables were found to relate to any of the insect feeding or growth variables when considered across all treatments and maternal plants. Nevertheless, a negative interaction between feeding by flea beetles and subsequent feeding by hornworms was supported.

TABLE 2. MEANS AND MANCOVA RESULTS FOR EFFECT OF PLANT TREATMENT AND MATERNAL PLANT ON LEAF CHEMISTRY

A. Means ± standard errors for leaf chemical variables[a]

Treatment[b]	TGA	PHEN	PROT	TI	POD	PPO
C	0.4200	102.51	52.183	0.9285	46.400	3.3318
	±0.0212*a*	±7.55*a*	±4.379*a*	±0.0717*a*	±7.543*a*	±0.3735*a*
W	0.4569	90.07	46.572	0.8251	52.222	3.9491
	±0.0369*a*	±7.88*a*	±3.076*a*	±0.0617*a*	±7.136*a*	±0.5037*a*
N	0.4885	162.36	96.803	0.8498	43.264	2.6723
	±0.0279*a*	±11.51*b*	±5.222*b*	±0.0647*a*	±6.045*a*	±0.3506*b*
NW	0.4677	153.50	85.534	0.9217	42.301	2.6614
	±0.0189*a*	±13.30*b*	±4.803*b*	±0.0712*a*	±6.397*a*	±0.2578*b*

B. MANCOVA: Test of overall effects

Source	Pillai's Trace	F	Hypothesis DF	Error DF	P
Intercept	0.979	588.55	6	75	0.000
Treatment	0.657	3.60	18	231	0.000
Clone[c]	0.661	1.10	54	480	0.298
T*C	1.598	1.08	162	480	0.278

C. MANCOVA: Tests of between-subjects effects

Source	Dependent variable	*df*	*f*	*p*
Intercept	TGA	1	1117.61	0.000
	PHEN	1	588.90	0.000
	PROT	1	1112.14	0.000
	TI	1	696.22	0.000
	POD	1	187.28	0.000
	PPO	1	301.86	0.000
Treatment	TGA	3	1.09	0.356
	PHEN	3	11.90	0.000
	PROT	3	34.26	0.000
	TI	3	0.60	0.618
	POD	3	0.44	0.724
	PPO	3	2.88	0.041
Clone	TGA	9	0.81	0.609
	PHEN	9	0.98	0.461
	PROT	9	0.91	0.519
	TI	9	1.75	0.091
	POD	9	0.43	0.914
	PPO	9	2.02	0.048
T*C	TGA	27	0.98	0.506
	PHEN	27	0.91	0.602
	PROT	27	1.51	0.082
	TI	27	0.84	0.695
	POD	27	1.29	0.193
	PPO	27	1.11	0.347

[a] Identical letters following data indicate values not differing significantly based upon one-way ANOVA.; TGA = total glycoalkaloid ($mg \cdot g^{-1}$), PHEN = total phenolics ($mg \cdot g^{-1}$), PROT = total protein ($mg \cdot g^{-1}$), TI = trypsin inhibitor activity (ug TI mg extract $protein^{-1}$), POD = peroxidase activity (Δ A_{470} $min^{-1} \cdot$ mg extract $protein^{-1}$), PPO = polyphenol oxidase activity (Δ $A_{470} min^{-1} \cdot$ mg extract $protein^{-1}$).

[b] Treatment = control (C), water (W), nitrogen (N), and nitrogen plus water (N + W).

[c] Clone = maternal plant source (Table 1).

TABLE 3. ANOVA AND ANCOVA RESULTS FOR OUTDOOR HORNWORM TRIALS[a]

Source	*df*	*f*	*p*
A. ANOVA: Dependent variable = RCR			
Intercept	1	654.89	0.000
Treatment (T)	3	0.83	0.491
Clone (C)	9	0.28	0.974
T*C	27	1.01	0.469
B. ANCOVA: Dependent variable = RCR			
Intercept	1	0.59	0.446
TGA	1	0.00	0.982
PHEN	1	0.75	0.391
PROT	1	0.77	0.383
TI	1	0.00	0.956
POD	1	0.01	0.945
PPO	1	0.44	0.508
Treatment (T)	3	1.22	0.316
Clone (C)	9	0.40	0.922
T*C	27	0.94	0.563

[a] For definitions of variables, see Table 2. RCR = relative consumption rate ($g \cdot g^{-1}$).

Effects on Fitness. Our estimate of short-term plant fitness (total fruit mass) was strongly affected by both plant treatment and maternal plant (Table 7A,B). The effect of plant treatment was associated with a nearly doubling of total fruit mass in nitrogen treated plants, with little or no effect due to water treatment (Table 7C). Upon incorporation of the leaf chemical variables and our estimate of flea beetle damage into the ANCOVA, only leaf PPO was found to relate significantly ($P < 0.05$) to total fruit mass (Table 7D), the effect being slightly positive based upon plots of residuals (not shown). Based upon the lack of significant correlations (results not shown), none of the leaf chemical parameters was associated with variation in plant growth and reproductive parameters. The clones did not differ in above-ground dry mass, but they did differ in total fruit number, average fruit mass, and total fruit mass (Table 7B). Thus, while clones differed in some aspects of growth and reproduction, virtually none of this variation could be associated with the leaf chemical and herbivory parameters measured in this study. Clones derived from distant sources (e.g., N2O, SERC, SEN) performed as well or better than clones derived locally (e.g., TIFT, AUG; Table 7B).

DISCUSSION

This study was designed to address questions related to the effect of plant nutrient variation on both leaf chemical allocation and effects of induced defenses.

TABLE 4. ANOVA, ANCOVA, AND REPEATED MEASURES RESULTS FOR INDOOR HORNWORM FEEDING TRIALS

Source	*df*	*f*	*p*
A. ANOVA: Dependent variable = total RCR			
Intercept	1	122.84	0.000
Treatment (T)	3	0.58	0.251
Clone (C)	9	0.47	0.353
T*C	27	0.40	0.524
B. ANCOVA: Dependent variable = total RCR			
Intercept	1	16.38	0.000
TGA	1	0.03	0.854
PHEN	1	4.00	0.049
PROT	1	0.09	0.771
TI	1	0.02	0.889
POD	1	0.43	0.515
PPO	1	1.23	0.271
Treatment (T)	3	0.51	0.680
Clone (C)	9	0.97	0.487
T*C	27	1.08	0.387
C. Repeated Measures ANOVA: Dependent variable = RCR			
Intercept	1	221.99	0.000
Treatment (T)	3	0.68	0.567
Clone (C)	9	0.72	0.685
T*C	27	1.23	0.235
D. Repeated Measures ANCOVA: Dependent variable = RCR			
Intercept	1	18.34	0.000
TGA	1	0.15	0.702
PHEN	1	3.27	0.075
PROT	1	0.01	0.938
TI	1	0.73	0.397
POD	1	1.27	0.264
PPO	1	1.12	0.293
Treatment (T)	3	0.62	0.607
Clone (C)	9	0.54	0.843
T*C	27	1.35	0.160
E. Repeated Measures ANOVA: Dependent variable = RGR			
Intercept	1	84.25	0.000
Treatment (T)	3	0.49	0.691
Clone (C)	9	0.62	0.770
T*C	27	1.43	0.113
F. Repeated Measures ANCOVA: Dependent variable = RGR			
Intercept	1	0.17	0.682
TGA	1	0.02	0.885
PHEN	1	0.00	1.000
PROT	1	0.42	0.518
TI	1	1.99	0.163
POD	1	0.02	0.891
PPO	1	1.30	0.257
Treatment (T)	3	0.50	0.686
Clone (C)	9	0.71	0.695
T*C	27	1.43	0.116

[a] For definitions of variables, see Table 2. RCR = relative consumption rate (g/g^{-1}); RGR = relative growth rate (g/g^{-1}).

TABLE 5. ANOVA AND ANCOVA RESULTS FOR FLEA BEETLE DAMAGE[a]

Source	*df*	*f*	*p*
A. ANOVA: Dependent variable proportion of leaf removed			
Intercept	1	883.82	0.000
Treatment (T)	3	0.67	0.577
Clone (C)	9	1.33	0.268
T*C	27	0.88	0.632
B. ANCOVA: Dependent variable proportion of leaf removed			
Intercept	1	29.14	0.000
TGA	1	0.13	0.722
PHEN	1	1.75	0.190
PROT	1	0.17	0.680
TI	1	0.02	0.894
POD	1	3.80	0.055
PPO	1	0.44	0.507
Treatment (T)	3	0.57	0.641
Clone (C)	9	1.19	0.338
T*C	27	0.90	0.614

[a] For definitions of variables, see Table 2.

Clear support for the CNB hypothesis is lacking in our results. Despite strong effects of supplemental nitrogen on plant size and reproduction, only leaf protein increased in nitrogen-treated plants as predicted by CNB. Leaf total phenolics increased in nitrogen-treated plants, while most other nitrogen-based metabolites were unaffected (POD, TI, TGA) or decreased (PPO). Herbivory was also

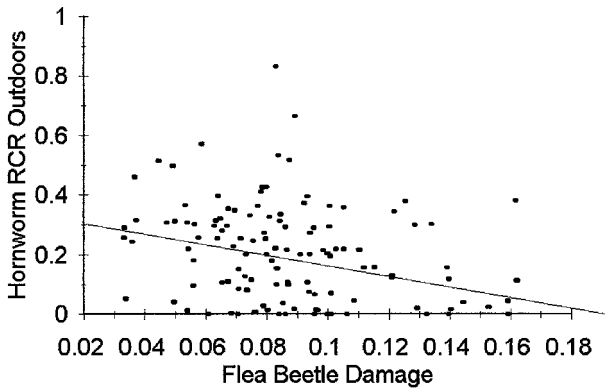


FIG. 1. The relationship between flea beetle damage (proportion of leaf area removed) and tobacco hornworm relative consumption rate (RCR; g dry leaf mass g wet larval mass) in outdoor feeding trials. The fitted line represents the least-squares linear regression of RCR on flea beetle damage.

TABLE 6. PEARSON CORRELATIONS AMONG LEAF CHEMICAL AND INSECT FEEDING VARIABLES[a]

Variable	Outdoor hornworm RCR	Indoor total hornworm RCR	Indoor total hornworm RGR	Flea beetle damage
TGA	−0.003	−0.088	−0.014	0.058
	0.977	0.341	0.876	0.531
PHEN	0.087	−0.200	−0.108	−0.142
	0.342	0.029	0.239	0.122
PROT	0.027	−0.172	−0.085	−0.002
	0.767	0.060	0.355	0.982
TI	−0.088	−0.047	−0.089	0.007
	0.336	0.609	0.334	0.936
POD	0.041	−0.033	0.023	−0.113
	0.659	0.723	0.805	0.218
PPO	0.066	−0.066	0.034	−0.007
	0.475	0.473	0.712	0.941
Outdoor	-	0.198	0.188	−0.276
hornworm RCR		0.030	0.040	0.002*
Indoor total	-	-	0.349	−0.073
hornworm RCR			0.000*	0.428
Indoor total	-	-	-	−0.116
hornworm RGR				0.206

[a] Data are correlation coefficients, followed by significance level (P values). Asterisks indicate significant effects ($P \leq 0.05$) following adjustment for multiple correlations using Bonferroni adjustment (alpha level was lowered to 0.002 for each test to bring the table-wide alpha level to 0.05.). For definitions of independent variables, see Table 2. Outdoor hornworm RCR = g · dry leaf mass g · wet larval mass^{-1}; Indoor total hornworm RCR = g · wet leaf mass g · wet larval mass^{-1}; Indoor total hornworm RGR = g · wet leaf mass g · wet larval mass^{-1}; Flea beetle damage = proportion of leaf area removed.

unaffected by plant treatment. This mixed bag of results is not entirely surprising, because some studies have shown full support of CNB (e.g., Bryant et al., 1993; Cipollini et al., 1993; Gebauer et al., 1998; Hoffland et al., 2000), numerous others mixed support (e.g., Reichardt et al., 1991; Lavola and Julkunen-Tiitto, 1994; Ruohomaki et al., 1996; Kinney et al., 1997; Keinanen et al., 1999; Mutikainen et al., 2000), and still others no support at all (e.g., Baldwin et al., 1993; Iason and Hester, 1993; Feller, 1995; Kainulainen, 1996). These inconsistent results have left some ecologists wondering if the hypothesis has any intrinsic value in terms of establishing a predictive framework (e.g., Hamilton et al., 2001). Still others have criticized "general defense theories" as a whole (e.g., Stamp, 1992; Haukioja et al., 1994; Langenheim, 1994), in that most studies require specific knowledge of the study system and much *post-hoc* qualification when interpreting results.

One hypothesis with potential for placing such diverse studies in a general framework is the OD hypothesis (Rhoades, 1979; Berryman 1988). Although some of its assumptions are hard to quantify (e.g., the fitness value of a plant part,

TABLE 7. ANOVA AND ANCOVA RESULTS FOR EFFECTS OF PLANT TREATMENT AND MATERNAL PLANT ON GROWTH AND REPRODUCTIVE VARIABLES

A. Means ± standard errors for growth and reproductive variables by treatment[a]

Treatment	ABDMASS	FRUITS	AVFTMASS	FTMASS
C	10.0 ± 0.8a	23.3 ± 5.7a	1.34 ± 0.08a	31.1 ± 1.3a
W	10.3 ± 0.8a	21.7 ± 3.8a	1.59 ± 0.13b	34.6 ± 1.5a
N	23.0 ± 1.5b	49.5 ± 5.2b	1.26 ± 0.08a	62.3 ± 1.1b
NW	22.2 ± 1.2b	57.9 ± 8.0b	1.67 ± 0.12b	96.9 ± 1.5b

B. Means ± standard errors for growth and reproductive variables by maternal clone[a]

Clone	ABDMASS	FRUITS	AVFTMASS	FTMASS
AUG	16.9 ± 2.1a	45.8 ± 10.3a	1.24 ± 0.07b	56.7 ± 7.4a
EAST	15.5 ± 2.4a	33.8 ± 11.9a	1.13 ± 0.13b	38.2 ± 8.7b
HOW	16.0 ± 2.4a	36.9 ± 6.4a	1.70 ± 0.07a	62.7 ± 7.8a
KENT	18.9 ± 3.0a	38.0 ± 8.9a	1.49 ± 0.12b	56.6 ± 10.4a
N2O	19.0 ± 2.7a	45.6 ± 11.3a	1.59 ± 0.16b	72.5 ± 11.9a
SACC	18.9 ± 3.2a	41.8 ± 11.0a	1.42 ± 0.14b	59.4 ± 10.1a
SEN	14.6 ± 2.0a	34.8 ± 14.9a	1.94 ± 0.28a	67.5 ± 16.1a
SERC	14.3 ± 2.2a	46.1 ± 7.6a	1.89 ± 0.20a	87.1 ± 10.9a
TIFT	15.1 ± 2.4a	36.8 ± 12.9a	1.10 ± 0.09b	40.5 ± 9.5b
TWIN	14.6 ± 2.5a	21.3 ± 6.2b	1.15 ± 0.14b	24.5 ± 6.0b

C. ANOVA: Dependent variable = total fruit mass (g)

Source	*df*	*F*	*P*
Intercept	1	108.29	0.000
Treatment (T)	3	5.87	0.003
Clone (C)	9	3.30	0.008
T*C	27	0.95	0.550

D. ANCOVA: Dependent variable = total fruit mass (g)

Source	*df*	*F*	*P*
Intercept	1	10.91	0.001
TGA	1	0.15	0.704
PHEN	1	0.00	0.980
PROT	1	0.13	0.719
TI	1	0.02	0.883
POD	1	0.02	0.892
PPO	1	4.12	0.046
Flea beetle damage	1	1.13	0.291
Treatment (T)	3	4.72	0.007
Clone (C)	9	3.14	0.010
T*C	27	0.99	0.492

[a] ABDMASS = aboveground dry mass (g), FRUITS = total fruit number per plant, AVFTMASS = average wet mass per fruit, FTMASS = total wet fruit mass per plant (g). Identical letters following data indicate values not differing significantly based upon one-way ANOVA.

the fitness value of allocation to secondary metabolites, the "costs" of secondary metabolites) and constraints exist that make predictions complex (Berenbaum and Zangerl, 1988; Linhart, 1991), it is a hypothesis grounded firmly in evolutionary theory. In general, this hypothesis states that plants should allocate resources to secondary metabolites in ways that maximize fitness—the exact ways will depend upon the biology, history, and ecology of the system being studied. With reference to our study, it is possible that nitrogen supplementation increased dietary protein content in leaves, as leaf and whole plant growth was promoted. Increased dietary protein content may have increased the need for antiherbivore defense (due to potentially increased risk of herbivory in protein rich leaves; e.g., Hare, 1987). As a result, phenolic production may have been increased via the up-regulation of enzymes such as POD (as we observed) or phenylalanine ammonia lyase (PAL), which may be a key regulatory factor affecting quantitative variation in total phenolics (Bate et al., 1994; Howles et al., 1996; Jones and Hartley, 1999). The net result would be little difference in levels of insect herbivory in N-treated and control leaves; this is what we found. In a similar vein, our results support the concept of a trade-off between growth/fitness and defense production. Untreated plants were half as large and produced half as many fruits as nitrogen-treated plants, yet produced similar levels of defense compounds and had similar levels of herbivory. It is possible that the maintenance of antiherbivore defense in untreated plants came at the expense of growth and fitness.

Based upon CNB hypothesis, we predicted that glycoalkaloids would increase in response to nitrogen supplementation and that insect feeding would decrease as a result; neither effect was observed. While nonadapted insects seem to be susceptible to glycoalkaloids (e.g., Hare, 1983, 1987; Fragoyiannis et al., 1998), leaf glycoalkaloids are apparently not commonly associated with resistance to specialized insects such as hornworms and flea beetles (Flanders et al., 1992) or to specialized leaf pathogens (Hoffland et al., 1999). In fact, TGA levels in *S. carolinense* leaves are relatively low compared with many other *Solanum* species (Cipollini et al., 2001), possibly suggesting a diminished defensive role for leaf glycoalkaloids in this plant species. While allocation of glycoalkaloids to roots was not measured in this study, it is known that levels of TGA are much higher in roots than in leaves (R. Walls, M. Cipollini, H. Appel, J. Schultz, unpublished data), where a functional role may be important. The lack of an effect of TI on hornworms may have a similar explanation; insect responses to TI may depend upon the degree of specialization of the insect on the host (Broadway, 1995). This pattern seems to be common in studies that have focused on specialized herbivores. This may be because the host and herbivore share a long evolutionary history with resultant "molecular modeling" (sensu Wink, 1998, 1999) of defense and resistance traits, or it may be due to physiological, ecological, or environmental constraints on genetically based chemical responses to herbivory—the "syntheses, synergisms, and sundry other sins" of Berenbaum and Zangerl (1988). In the latter

case, negative effects on specialized herbivores may be seen in some circumstances and not others.

With respect to our second and third questions, which relate to induced defenses, we hypothesized that flea beetles might induce plant defenses and, thus, negatively impact hornworm feeding. Based upon our results, we can only say that a negative relationship was supported, but the source of this negative relationship remains to be identified. In our field experiment, the level of flea beetle damage was not experimentally controlled; rather, flea beetles were allowed to consume plants prior to our observations of leaf chemistry and hornworm feeding. We assumed that leaf chemistry might have changed between the time flea beetles attacked and the time leaves were analyzed; thus, all measured chemical variables should be considered potentially induced in this study. Acknowledging this, patterns of flea beetle feeding might have partly reflected differences in leaf chemistry prior to feeding, and subsequent induction may have produced a saturated response in the plants (because overall flea beetle damage was high). In any case, this is a problem common to field studies that attempt to establish correlations among variables, and at the very least the study addresses these effects within a realistic ecological context. Hence, we can only speculate about the source of the negative interaction between flea beetle damage and hornworm feeding. One possibility is that other leaf chemical or physical defenses (e.g., toughness, thorniness) or signals (e.g., volatiles) might have been induced in plants heavily infested with flea beetles. A second possibility is that the flea beetle damage to leaves may have simply made it physically more difficult for hornworms to efficiently process damaged leaf material, thus slowing consumption rates. Pieces of unconsumed leaf material were commonly encountered in the bottom of cups away from where the larvae stayed and fed (attached to the main branch). This negative relationship was present, but not significant, for the indoor hornworm trials. We surmise that conditions produced in the (less natural) indoor trials may have obscured the relationship identified in the field.

The lack of variation among plant clones was surprising, given the wide area from which they were originally derived. Parameters that showed significant variation among the clones were total fruit number, average fruit mass, and total fruit mass. Based upon total fruit mass (which we considered an estimate of short-term fitness), the two clones that showed highest fitness in 2000 were derived from maternal plants in Maryland (SERC, N2O), thus discounting the idea that adaptation to local (Georgia) conditions might be responsible for variation in plant performance. The effect of maternal clone was not involved in any significant interaction in any of the analyses, indicating that the clones generally responded similarly to treatments and/or herbivory, regardless of genetic background.

In conclusion, the results of this study provide insight into the effects of nitrogen supplementation and induction by herbivores on leaf secondary metabolites and herbivory in this species, but they provide little support for the CNB hypothesis.

Because plants seemed to maintain levels of resistance under low nitrogen conditions at the expense of growth and reproduction, our results show support for some aspects of the OD hypothesis. Future studies focusing on this study system might concentrate on identifying more precisely which secondary metabolites are responsible for the general responses we observed, which genes/enzymes are responsible for these responses, and specifically how these changes influence the common "generalist" herbivores of this plant species. We concur with others in suggesting that framing future studies in terms of the OD hypothesis might be a more productive and insightful approach, in comparison with continued focus on the CNB hypothesis. Our results further suggest that herbivory by one organism can influence herbivory by another via induced responses; however, the mechanism responsible for this effect remains to be discovered for our study system.

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RELATIONSHIP BETWEEN NUTRITIONAL COMPOSITION OF PLANT SPECIES AND INFESTATION LEVELS OF THRIPS

ALISON S. SCOTT BROWN,[1,2] MONIQUE S. J. SIMMONDS,[1,*] and WALTER M. BLANEY[2]

[1]*Royal Botanic Gardens*
Kew, Surrey, TW9 3AB, United Kingdom

[2]*School of Biological and Chemical Sciences*
Birkbeck College, University of London
Malet Street, London, WC1E 7HX, United Kingdom

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Abstract—Levels of soluble protein and carbohydrate (raffinose, sucrose, glucose, and fructose) in leaves from a selection of plant species were measured to determine if a relationship existed between these nutrients and infestation by *Frankliniella occidentalis* and *Heliothrips haemorrhoidalis*. Most species of host plant examined contained a higher proportion of protein than carbohydrates, and overall, leaves from species of plants that supported populations of thrips had greater levels of protein than leaves from nonhost species. New leaves and flowers that supported *F. occidentalis* contained high levels of carbohydrate and protein. The quantity of protein in leaves at the top of the tree, *Peumus boldus*, was greater than in leaves from lower levels, and the amount of feeding damage accrued by *H. haemorrhoidalis* was greater on the upper foliage than lower foliage. Oviposition by *H. haemorrhoidalis* was positively correlated to levels of protein in host plants but not to levels of carbohydrates. Overall, levels of soluble protein in plants influenced their susceptibility to thrips more than levels of carbohydrates.

Key Words—Carbohydrate, protein, sucrose, fructose, glucose, raffinose, *Frankliniella occidentalis*, *Heliothrips haemorrhoidalis*, host plant, nutrition, oviposition, feeding damage.

INTRODUCTION

In the last decade, thrips have become of increasing importance as pests, especially in protected crops and in large glasshouses containing botanical collections.

* To whom correspondence should be addressed. E-mail: m.simmonds@rbgkew.org.uk

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Although researchers have started to gather data about the factors that influence the host selection behavior of thrips, we do not understand fully the impact of primary and secondary metabolites in the host selection of invasive, polyphagous species such as *Frankliniella occidentalis* (Pergande) and *Heliothrips haemorrhoidalis* (Bouché). The ratio of protein to carbohydrate in plants has been reported to affect the performance of species of herbivorous insect (Schoonhoven et al., 1998a). Increased protein quantity is associated with enhanced herbivore survival, growth, and fecundity (McNeill and Southwood, 1978; Mattson 1980; Ohgushi, 1992; Slansky, 1993), although the quality of plant proteins may affect the levels of insect performance (Karowe and Martin, 1989). Research has shown that most herbivorous insects use carbohydrates as feeding stimulants (Bernays and Simpson, 1982; Blaney and Simmonds, 1988) and as important nutrients needed to synthesize body tissue and serve as energy sources (Schoonhoven et al., 1998b). Sugars have also been shown to promote oviposition in some species (Derridji et al., 1996).

Scott Brown (2002) showed that species of plants selected by thrips from among the botanical collection held in the Temperate House, Royal Botanic Gardens, Kew (RBG, Kew), varied in their suitability to support populations of *H. haemorrhoidalis* and *F. occidentalis*. The performance criteria examined included longevity, feeding and oviposition levels, growth, and development of thrips on individual leaves of these plant species. In this study, we test the hypothesis that protein levels in plants will influence the suitability of plants as hosts for thrips more than levels of carbohydrates. We also investigate whether the quantity of carbohydrates and proteins present in foliage influences the oviposition performance of *H. haemorrhoidalis*. In addition, the distribution of soluble proteins and carbohydrates in the foliage of a tall tree, *Peumus boldus* Molina (Monimiaceae), was examined to determine if the location of *H. haemorrhoidalis* on the tree related to the nutritional levels in the leaves.

METHODS AND MATERIALS

Carbohydrate Analysis

In the majority of species of plants, the proportion of soluble carbohydrate consists mainly of sucrose, glucose, and fructose with minor quantities of other compounds such as aminosugars, sugar alcohols, phosphates, and uronic acids (Gonzalez et al., 1989). Four sugars were selected for quantification, representing three sugar categories: oligosaccharides (raffinose), disaccharides (sucrose), and monosaccharides (glucose and fructose). Analysis of sugars in leaf extracts was performed directly using high-pressure liquid chromatography (HPLC).

Preparation of Extracts. Extracts were made up using 50 mg of air dried leaf material from species of plants growing in the Temperate House, RBG, Kew, UK (Scott Brown et al., 1999) (Table 1). Finely ground plant material was placed in

TABLE 1. LEVELS OF PROTEINS AND CARBOHYDRATES (MEAN ± SEM) IN LEAF SAMPLES COLLECTED FROM SELECTED SPECIES OF PLANTS

Plant species[a]	Plant family	Plant tissue[b] P/C	Host status[c]	Protein (mg/g, mean ± SE)	Protein (%)[d]	Total carbohydrate (mg/g, mean ± SE)[e]	Raffinose (mg/g, mean ± SE)	Sucrose (mg/g, mean ± SE)	Glucose (mg/g, mean ± SE)	Fructose (mg/g, mean ± SE)	Protein: carbohydrate ratio[f]
Dombeya acutangula Cav.	Sterculiaceae	leaf (new) $N = 5/N = 4$	Hh & Fo	99.8 ± 6.85	10.0	142.5 ± 3.38*	8.0 ± 2.01	60.8 ± 1.61	35.6 ± 2.83*[g]	38.2 ± 3.24	41:59 B
D. acutangula		leaf (mature) $N = 5/N = 4$	Hh & Fo	72.8 ± 4.29	7.3	91.7 ± 6.92	0	48.9 ± 3.50	19.6 ± 3.30	23.3 ± 5.41	44:56 B
Tephrosia grandiflora Pers.	Leguminosae	leaf (new) $N = 4/N = 4$	Hh & Fo	185.5 ± 6.07	18.6	29.2 ± 2.37	2.6 ± 1.05	17.5 ± 0.52	5.1 ± 0.68	4.0 ± 0.72	86:14 A
T. grandiflora		leaf (mature) $N = 4/N = 4$	Hh & Fo	80.2 ± 0.68	8.0	28.6 ± 0.30	0.3 ± 0.26	20.4 ± 0.34*	4.2 ± 0.71	3.7 ± 0.03	74:26 A
Cryptocarya alba R.Br.	Lauraceae	leaf (new) $N = 15/N = 12$	Hh	207.0 ± 42.64	20.7	79.8 ± 9.36*	16.7 ± 8.24	24.3 ± 6.05	27.3 ± 3.54*	11.7 ± 1.61	72:28 A
C. alba		leaf (mature) $N = 12/N = 12$	Hh	107.3 ± 6.39	10.7	50.9 ± 8.19	11.2 ± 3.9	13.4 ± 3.28	16.4 ± 3.78	9.9 ± 1.45	68:32 A
Peumus boldus Molina	Monimiaceae	leaf (new) $N = 15/N = 12$	Hh	63.2 ± 7.21	6.3	77.4 ± 15.93	8.0 ± 5.59	47.0 ± 11.70	11.4 ± 2.14	11.0 ± 1.42	45:55 B
P. boldus		leaf (mature) $N = 12/N = 12$	Hh	43.8 ± 6.35	4.4	70.7 ± 7.78	14.5 ± 4.35	33.5 ± 5.36	13.4 ± 1.18	9.8 ± 1.08	46:54 C
Erica canaliculata L.	Ericaceae	leaf (new) $N = 4/N = 4$	Hh	166.1 ± 5.86	16.6	51.3 ± 13.09	12.4 ± 11.42	23.1 ± 1.52*	6.7 ± 0.15	9.1 ± 0.36	76:24 A
E. canaliculata		leaf (mature) $N = 4/N = 4$	Hh	110.4 ± 15.54	11.0	40.5 ± 3.51	5.7 ± 3.75	3.7 ± 0.32	22.6 ± 4.63*	8.5 ± 3.0	73:27 A
Saurauia nepaulensis Willd.	Actinidiaceae	leaf (mature) $N = 4/N = 4$	Hh	64.5 ± 0.92	6.5	26.3 ± 0.48*	0.6 ± 0.32	4.4 ± 0.2*	8.8 ± 0.43*	12.5 ± 0.61	71:29 A
S. nepaulensis		leaf (new) $N = 4/N = 4$	Hh	63.1 ± 4.6	6.3	19.7 ± 0.51	0	2.0 ± 0.20	4.8 ± 0.48	12.9 ± 0.64	76:24 A
Inga insignis Mill.	Leguminosae	leaf (new) $N = 4/N = 1$	Hh	55.1 ± 2.14	5.5	25.3 ± 0	5.1 ± 0	16.9 ± 0	3.3 ± 0	0	69:31 D
Greyia radlkoferi Hook and Harv.	Greyiaceae	leaf (new) $N = 4/N = 4$	Hh	158.5 ± 14.65	15.9	23.5 ± 5.47	0	4.4 ± 2.74	6.1 ± 3.08	13.0 ± 3.41	87:13 A
G. radlkoferi		leaf (mature) $N = 6/N = 4$	Hh	227.8 ± 9.15	22.8	21.9 ± 5.8	0	2.8 ± 0.96	8.5 ± 1.36	10.5 ± 3.5	91:9 A
Crotalaria capensis L.	Leguminosae	flower $N = 4/N = 4$	Fo	108.2 ± 2.34	10.8	75.3 ± 2.18	0.4 ± 0.40	52.6 ± 2.31	7.8 ± 1.08	14.5 ± 0.95	59:41 B
C. capensis		leaf (mature) $N = 4/N = 4$	Fo	88.6 ± 5.26	8.9	40 ± 1.33*	2.3 ± 0.84	31.1 ± 1.44*	0	4.6 ± 0.29	69:31 A
C. capensis		leaf (new) $N = 4/N = 4$	Fo	100.6 ± 8.14	10.1	27.4 ± 3.0	5.7 ± 0.90	20.3 ± 1.14	0	3.8 ± 0.39	79:21 A
Crambe strigosa L.	Cruciferae	leaf (mature) $N = 4/N = 1$	Fo	67.1 ± 4.69	6.7	47.4 ± 0	2.5 ± 0	28.9 ± 0	4.1 ± 0	11.8 ± 0	59:41 D
C. strigosa		leaf (new) $N = 4/N = 1$	Fo	65.8 ± 19.58	6.6	34.3 ± 0	1.9 ± 0	26 ± 0	0	6.5 ± 0	66:34 D
Capsicum annuum L.	Solanaceae	leaf (mature) $N = 4/N = 4$	Fo	94.3 ± 3.38	9.4	19.5 ± 1.5	3.5 ± 0.09	6.5 ± 0.71	3.5 ± 0.42	6.1 ± 0.5	83:17 A
Justicia adhatoda L.	Acanthaceae	leaf (new) $N = 4/N = 1$	Fo	101.1 ± 1.74	10.1	16.1 ± 0	1.8 ± 0	8.5 ± 0	2.1 ± 0	3.6 ± 0	86:14 D
Alsinidendron trinerve H. Mann.	Caryophyllaceae	leaf (new) $N = 4/N = 4$	Fo	93.9 ± 1.37	9.4	7.1 ± 0.22	0	6.8 ± 0.30	0.3 ± 0.31	0	93:7 A
Sclerochiton harveyanus Harv.	Acanthaceae	leaf (mature) $N = 4/N = 4$	N-H	74.1 ± 10.29	7.4	42.4 ± 3.63	3.5 ± 1.5	11.7 ± 3.27	14.7 ± 0.46	12.6 ± 1.37	64:36 B
Gardenia posoquerioides Ellis	Rubiaceae	leaf (mature) $N = 4/N = 1$	N-H	32.0 ± 5.21	3.2	29.3 ± 0	25.9 ± 0	3.4 ± 0	0	0	52:48 D
Pavonia praemorsa Cav.	Malvaceae	leaf (mature) $N = 4/N = 4$	N-H	75.8 ± 0.49	7.6	21.6 ± 2.22	1.2 ± 0.7	14.2 ± 2.17	3.6 ± 0.37	2.6 ± 1.28	78:22 A
Veltheimia viridifolia Gled.	Hyacinthaceae	leaf (mature) $N = 4/N = 1$	N-H	50.4 ± 0.70	5.0	21.1 ± 0	2.0 ± 0	13.5 ± 0	2.7 ± 0	2.9 ± 0±)	71:29 D

[a] Leaf samples are listed in order of decreasing total carbohydrate values, per species associated with host status.
[b] Leaf (age) or flower, number of replicates protein/carbohydrate.
[c] Host status, Hh = *H. haemorroidalis*, Fo = *F. occidentalis*, N-H = non-host.
[d] Percentage soluble protein calculated from amount of protein in leaves mg/g.
[e] Total carbohydrate = sum total amount of raffinose, sucrose, glucose, fructose.
[f] Protein:Carbohydrate ratio (% of total C + P), where A = higher protein content than carbohydrate ($P < 0.05$), B = similar quantities of protein and carbohydrate, C = higher carbohydrate than protein ($P < 0.05$), D = no statistical comparison available (carbohydrate, $n = 1$).
[g] Asterisk Indicates significantly higher levels of sugars in new or mature leaves of plants of the same species (MW, $P < 0.05$).

a 10-ml glass vial with 4 ml of 80% ethanol and sealed with a screw lid. Vials were placed in a blood-tube mixer (Stuart Scientific) and left rotating at the vertical setting for 16 hr at 4°C. The lid of each vial was released before the vial was placed in a water bath (90°C) for 10 min. Extracts were removed from the water bath and spun in a centrifuge (13,000 rpm for 10 min). The supernatant was removed from each vial with a Pasteur pipete and collected in 5-ml vials and reduced under a nitrogen air stream. The concentrated extract was frozen and freeze-dried. Extracts were taken up in 500 μ1 double-distilled H_2O and mixed thoroughly. Each extract was transferred to an Eppendorf tube and centrifuged (13,000 rpm for 10 min), before being injecting into the HPLC system.

Quantitative Measurement of Primary Sugars Using HPLC. The quantification of sucrose, fructose, glucose, and raffinose in leaf extracts was undertaken using a Waters HPLC system. The mobile phase was a dilute buffer of sodium sulfate (0.01 M) with a flow rate of 0.6 ml/min. Each sample was injected into a 20-μl loop and passed initially through a guard column (Phenomenex) before entering a sodium based carbohydrate column (Phenomenex) maintained at a temperature of 65°C. The separated sugars were detected using a Waters R-400 differential refractometer and recorded by a Waters 745B integrator. A known range of sugar standards, 0.1–10 mg/ml (BDH, Poole, UK) was used to construct a standard reference curve.

Protein Analysis

The most valuable proteins in plants, in terms of nutrition, tend to be the soluble enzymes that are easy for insects to extract and digest (Bernays and Chapman, 1994). This study considered the quantification of soluble protein present in leaf samples of the selected plant species as a measure of nutritional value as hosts of thrips. The bicinchoninic acid protein assay (BCA) (Pierce Chemical, 1996) was used to quantify the amount of soluble protein content of leaf samples in a method described by Smith et al. (1985).

Preparation of Extracts. Leaves were collected from plant specimens located within the Temperate House, RBG, Kew (Table 2) and air dried. Ground leaf material (10 mg) was placed into an Eppendorf tube with 1.5 ml of Tris HCl buffer (pH 8) and mixed with a vortex mixer. The Eppendorf tube was placed in a rotator overnight at 4°C. After centrifuging the Eppendorf tube (13,000 rpm) for 10 min, the supernatant was decanted into a clean tube through Whatman No. 1 filter paper (42.5 mm diam.) (Whatman). Compounds present in leaf extracts, which may interfere with the absorbency readings at 562 nm (Brown et al., 1989), were removed using acetone. Proteins in these samples were precipitated by adding 200 μl of acetone (−20°C) to 50 μl of extract in an Eppendorf tube (1.5 ml), which was thoroughly mixed and then stored at −20°C for 30 min. The extract–acetone solution was removed from the freezer and centrifuged at 13,000 rpm for 10 min. The

TABLE 2. DISTRIBUTION OF *H. haemorrhoidalis* FEEDING IN RELATION TO PROTEIN AND CARBOHYDRATE LEVELS IN FOLIAGE OF TREE SPECIES, *Peumus boldus*

	Nutritional analysis of foliage							Area of feeding damage (um^2)		
Peumus boldus' branch height[a]	Soluble protein (mg/g) $N = 5$	Raffinose (mg/g)	Sucrose (mg/g)	Glucose (mg/g)	Fructose (mg/g)	Total carbohydrate (mg/g) $N = 4$	Protein–carbohydrate ratio[c]	collection 1[d]	collection 2	collection 3
Top (5m)	75.5 (2.32)	13.0 (4.61)	37.9 (6.62)	12.7 (1.52)	8.2 (1.01)	71.8 (9.13)	51:49	420.3 (51.56)	289.3 (55.13)	293.4 (51.57)
Middle (3m)	37.0 (0.66)	9.5 (5.25)	41.3 (6.72)	16.6 (2.50)	11.9 (1.68)	79.2 (8.58)	32:68	395.2 (61.97)	182.8 (27.55)	134.7 (26.70)
Lower (1m)	19.0 (0.50)	10.0 (8.47)	27.9 (11.82)	8.3 (1.51)	10.6 (1.34)	56.8 (19.97)	25:75	62.21 (19.56)	59.8 (15.16)	33.6 (4.00)
Difference between crowns[b]	a, b, c (all $P < 0.05$)		a, b (all $P < 0.05$)	b ($P < 0.05$)		a, b (all $P < 0.05$)		a ($P < 0.001$) b ($P < 0.001$)	a ($P < 0.001$) b ($P < 0.001$)	a, b (all $P < 0.001$) c ($P < 0.05$)

[a] Leaves collected from branches at three height locations (top, middle and lower branches) of *Peumus boldus*.
[b] a = significantly higher levels in top crown than lower crown, b = significantly higher levels in middle crown than lower crown, c = significantly higher levels in top crown than middle crown (Mann-Whitney U test).
[c] Protein–carbohydrate ratio (% of total C + P).
[d] Feeding damage was examined on three different sampling dates; 1 = 10/01/96, 2 = 12/02/96, 3 = 11/03/96, $N = 20$ at each crown level.

acetone could then be poured off, and the protein pellet retained in the Eppendorf tube. A further 200 μl of acetone (-20°C) was added and well mixed with the pellet, and the process was repeated to ensure a clean protein sample. The protein pellet was taken up in 50 μl of double distilled water and mixed thoroughly using a vortex mixer before 1 ml of the bicinchoninic acid reagent solution was added (Smith et al., 1985). All tubes were incubated at 37°C for 30 min. The Eppendorf tubes were removed and cooled to room temperature for 15 min. Absorbency of each extract was then read at 562 nm against the reference sample.

Preparation of BSA Standards. Standards were prepared by diluting the 2.0 mg/ml bovine serum albumin (BSA) stock standard obtained from Pierce Chemical Company with Tris HCl buffer (pH8) (2000, 1500, 1000, 750, 500, 250, 125, and 25 μg/ml), and a standard curve constructed.

Relationship between Thrips Distribution and Protein and Carbohydrate Content of Foliage in a Tall Tree Species. A total of 60 leaves was collected from a Chilean tree, *Peumus boldus*, at three different height locations, top (5 m), middle (3 m), and lower (1 m). The area of feeding damage caused by thrips feeding was measured using a quantimet 520 image analysis system (Cambridge Instruments Ltd.), which, when coupled to a macroviewer, was used to detect the light patches on the leaf surfaces. Leaf collections were repeated on two further occasions for comparison. A further 20 leaves were taken at these crown levels and analyzed for levels of protein and carbohydrate as describe above.

Levels of Oviposition on Several Species of Plants Host to H. haemorrhoidalis. Leaves were excised from selected host plants, and the petiole was placed into a 10-cm glass vial containing distilled water. Vials containing single leaves were corked with cotton wool plugs and placed into Perspex boxes (22 cm long $\times$ 8 cm high $\times$ 12 cm deep, Stewart Plastics) that contained filter paper dampened with distilled water to minimize leaf desiccation.

Pseudopupal (pupae) stages of *H. haemorrhoidalis* were collected from the Australian tree fern, *Cyathea australis* Sm. (Cyantheaceae), situated in the Princess of Wales Conservatory, RBG, Kew. Five pupae were placed on individual leaves of the plant species to be examined, and the box was sealed with a lid and Parafilm® (American National Can™, USA). The box was stored at 27°C, 16L:8D for 3 days. After 3 days, eclosed adults were removed and placed onto fresh leaves of the same species and stored under the same conditions. Leaves were exchanged for fresh material at 7-day intervals for the duration of the time the adult thrips survived. The removed leaves were examined under the microscope in order to measure oviposition. Five to eight replicas for each host species examined were set up.

Analysis of Data

Data were analyzed using Kruskal-Wallis (KW) one-way analysis by ranks and the Mann-Whitney U test (MW), using two-tailed probability (Fowler et al., 1999).

RESULTS

Carbohydrate Levels. The quantity of each of the four carbohydrates varied among the species of plant examined (raffinose KW, $P < 0.01$; sucrose KW, $P < 0.001$; glucose KW, $P < 0.001$; fructose KW, $P < 0.001$; Table 1). Within some species, the levels of carbohydrates varied between young and mature leaves. For example, within *D. acutangula* and *C. alba*, the amounts of carbohydrates were greater in new leaves than mature leaves. In contrast, *S. nepaulensis* and *C. capensis* contained higher levels of carbohydrates in mature leaves relative to new leaves. In *C. capensis*, this was due to greater levels of sucrose, whereas in *S. nepaulensis* the levels of both sucrose and glucose were greater in the mature leaves.

Overall, higher concentrations of sugars were present in the leaves of hosts of *H. haemorrhoidalis* compared with hosts of *F. occidentalis* (KW, $P < 0.05$; Table 1). Host plants of *H. haemorrhoidalis* contained sucrose in greater abundance than glucose (MW, $P < 0.05$), fructose (MW, $P < 0.001$) or raffinose (MW, $P < 0.001$). Leaves of host plants of *F. occidentalis* were also found to contain more sucrose than any of the other three sugars quantified (MW, $P < 0.001$, in each case). The greatest level of carbohydrate was recorded in the young leaves of *D. acutangula* (MW, $P < 0.05$, in all cases): A host plant of both *F. occidentalis* and *H. haemorrhoidalis*. Flowers of *C. capensis*, a host of *F. occidentalis*, contained higher levels of carbohydrates than the leaves (MW, $P < 0.05$).

Protein Levels. The quantity of soluble protein in the foliage of the seventeen plant species varied among species (KW, $P < 0.001$; Table 1). Usually young flush leaves contained higher levels of protein than mature leaves from the same species (KW, $P < 0.05$), although mature leaves of *G. radlkoferi*, a host of *H. haemorrhoidalis*, contained higher levels of protein than occurred in the new leaves (MW, $P < 0.05$). Protein levels in hosts of *H. haemorrhoidalis* and *F. occidentalis* were similar (MW, $P > 0.05$) and, overall, were at higher levels than occurred in the non-hosts (KW, $P < 0.05$). However, the nonhost species *S. harveyanus* and *P. praemorsa* contained as much protein as *D. acutangula*, a host to both species of thrips (MW, $P > 0.05$, in both cases).

Ratio of Protein to Carbohydrate. The ratio of protein to carbohydrate in the foliage of the plants examined showed that most species (88%) contained a higher proportion of protein than carbohydrate (Table 1). The actual amounts of protein and carbohydrate were often greater in host plants than non-host plants.

Relationship between Thrips Distribution and Protein and Carbohydrate Content of Foliage in a Tall Tree Species. Feeding damage caused by *H. haemorrhoidalis* on the foliage of *P. boldus* varied among the three height levels, with the greatest levels of damage evident towards the top of the tree (Table 2). The proportion of protein in leaves was greater at the top of the tree than the bottom. Leaves of *P. boldus* collected from the upper crown contained higher levels

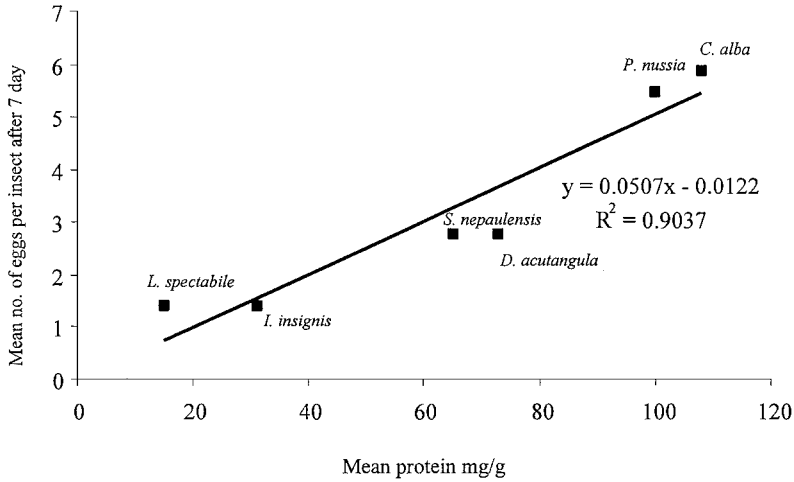


FIG. 1. Oviposition Levels of *H. haemorrhoidalis* in Relation to Soluble protein content in the leaves of six hosts plants.

of sucrose than the lower crown, with both sucrose and glucose being in greater concentrations in the middle crown compared with the lower crown.

Levels of Oviposition on Several Species of Plants Host to H. haemorrhoidalis. The amount of protein in the leaves of hosts of *H. haemorrhoidalis* correlated positively with the number of eggs laid by *H. haemorrhoidalis* ($r^2 = 0.9$; Figure 1). In contrast, oviposition was not correlated to the amount of carbohydrate in the leaves ($r^2 = 0.05$).

DISCUSSION

This investigation has shown that the ratio of soluble protein to carbohydrate (as represented by the four sugars, sucrose, glucose, fructose, and raffinose) in the foliage of 17 species of plants that support thrips varied from 93:7 (*Alsinidendron trinerve* a host for *F. occidentalis*) to 41:49 (*D. acutangula* a host to both *H. haemorrhoidalis* and *F. occidentalis*). The majority of host species contained a higher ratio of protein than carbohydrate, and generally the amounts of protein were greater in host species than nonhosts. These findings, along with the fact that the egg-laying behavior of *H. haemorrhoidalis* was positively correlated to protein content but not to carbohydrate content, support the hypothesis that protein influences the suitability of plants as hosts for thrips more than levels of carbohydrates.

Bernays and Chapman (1994) collated previous research on protein levels in plants and showed that protein and amino acid levels can range from 1% to 40% of the dry weight of foliage. Woody species in the Ericaceae and Myrtaceae contained

low levels of protein, whereas the greatest levels were recorded in herbaceous legumes. In our study, some of the greatest levels of soluble protein were recorded in the large shrubs and trees, such as *Greyia radlkoferi*, *Saurauia nepaulensis* and *Erica canaliculata*, all of which supported large infestations of *H. haemorrhoidalis* (Scott Brown, 2002).

Within a plant, levels of protein and carbohydrate vary and could influence the preferred feeding sites of the thrips. *F. occidentalis* feeds mainly on leaf buds, new leaves, and flowers (Trichilo and Leigh, 1988; Ullman et al., 1992) that usually contain higher levels of protein than the mature leaves (Bernays and Chapman, 1994). Our data show a trend for young leaves to have greater levels of protein than mature leaves, although on the two plants infested with both *F. occidentalis* and *H. haemorrhoidalis*, the levels of protein were higher in the new leaves. This would suggest that protein influences the host selection behavior of *F. occidentalis* more than *H. haemorrhoidalis*. The leaves of the tree *P. boldus* contained a high sugar content and only moderate levels of protein, yet this species suffered extensive damage due to thrips feeding in the Temperate House (Scott Brown, 2002).

Although rates of survival, reproduction, and development of herbivorous insects are linked to nitrogen levels in plants (Strong et al., 1984), sugars are important and stimulate feeding in some thrips (Boyce and Persing, 1939; Morse, 1995; Parella, 1995). However, it is unclear from our results what role carbohydrates play in host selection behavior of thrips. Levels of carbohydrate in plants did not correlate with the oviposition behavior of *H. haemorrhoidalis*. However, within the tall tree *P. boldus*, distribution of thrips at the top and middle of the tree was associated with high levels of carbohydrates, although protein levels were also higher in leaves from the top and middle of the tree. Therefore, is not clear whether the thrips are responding to the proportion of protein, carbohydrate, or both. Further research on a wider range of hosts as well as on diets that contain defined ratios of protein and carbohydrates are required to quantify the role soluble protein plays in the selection behavior and establishment of populations of *F. occidentalis* and *H. haemorrhoidalis*.

Low levels of primary metabolites or increased levels of secondary metabolites, such as alkaloids and tannins, are both factors that can affect plant selection as a food source for insects (Schoonhoven et al., 1998a). To date there is little information in the literature about the types of secondary compounds that could modulate the host selection behavior of thrips. Preliminary experiments using leaf-discs of species that had similar levels of protein and carbohydrates varied in their ability to support thrips (Scott Brown, 2002). This suggests that secondary metabolites in the leaves play a role in plant–thrips interactions. Identification of secondary metabolites in leaves of plant species that are unable to support thrips, such as *Sclerochiton harveyanus*, may provide a valuable lead for compounds that are active against *H. haemorrhoidalis* and *F. occidentalis*, and this will be the focus of further investigations.

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MONOTERPENE EFFECT ON FEEDING CHOICE BY DEER

GWENAËL VOURC'H,[1,4] MICHEL DE GARINE-WICHATITSKY,[2] ALICE LABBÉ,[2] DIMITRI ROSOLOWSKI,[1,3] JEAN-LOUIS MARTIN,[1,*] and HERVÉ FRITZ[3]

[1]*Centre d'Ecologie Fonctionnelle et Evolutive, CNRS UPR 9056*
1919 route de Mende
F-34293 Montpellier Cedex 5, France

[2]*Institut Agronomique néo Calédonien, CIRAD*
Station de Port-Laguerre, BP 25
Païta, 98890 New Caledonia

[3]*Centre d'Etudes Biologiques de Chizé, CNRS UPR 1934*
F-79360 Beauvoir-sur-Niort, France

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Abstract—A previous study showed that Sitka black-tailed deer (*Odocoileus hemionus sitkensis*) consumption was negatively correlated with monoterpene content in western redcedar (*Thuja plicata*). To test whether these monoterpenes were deterrent to Sitka black-tailed deer, we performed feeding choice experiments with four hydrocarbon (sabinene, myrcene, α-pinene, and $d + l$-limonene) and one oxygenated (α,β-thujone) monoterpene solution at their highest natural concentration in western redcedar foliage. To test whether deer response was species specific, we ran similar experiments on European roe deer (*Capreolus capreolus*) and rusa deer (*Cervus timorensis russa*). In all experiments, monoterpenes were repellent. Solutions with α,β-thujone, the major monoterpene in redcedar leaves, were the most repellent of the solutions tested. We then analyzed how black-tailed and roe deer responded to (1) an increase in concentration of the monoterpenes with the weakest repellent effects (hydrocarbon monoterpenes) and (2) a decrease in concentration of the monoterpene with strongest effect (α,β-thujone). Repellency tended to increase with concentration for hydrocarbon monoterpenes, but remained strong for α,β-thujone. As wild deer regularly feed on plants containing monoterpenes, this raises the question as to how the animals deal with these molecules.

Key Words—Monoterpenes, deer, cervids, feeding choice, herbivory.

* To whom correspondence should be addressed. E-mail: martin@cefe.cnrs-mop.fr
[4]Current address: Unité d'Epidémiologie Animale, INRA, 63122 Saint-Genès-Champanelle, France.

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INTRODUCTION

Monoterpenes, 10-carbon compounds biosynthesized from two isoprene (2-methyl-1,3-butadiene) units, are widely found as secondary metabolites in plants and are produced by nearly all conifers (Banthorpe and Charlwood, 1980; Bohlmann and Croteau, 1999; Trapp and Croteau, 2001). Numerous studies have shown that higher monoterpene concentrations in plants correlate with lower consumption by ungulate herbivores and, therefore, likely play an important role in the plant–herbivore interaction (Connolly et al., 1980; Personius et al., 1987; Duncan et al., 1994; Riddle et al., 1996; Estell et al., 1998a; Vourc'h et al., 2001). However, other studies have failed to find this correlation (Welch et al., 1983; Behan and Welch, 1985). To prove that monoterpenes are the cause of the deterrence of a plant, one needs to test their effects experimentally. We know of only three studies that have done so, using ungulate herbivore food-choice assays. The first, by Elliot and Loudon (1987), tested the effect of monoterpene odors on captive red deer calves (*Cervus elaphus*). The other two, by Estell et al. (1998b, 2000), tested the effect of monoterpenes on domestic sheep. These studies found that some monoterpenes are indeed deterrent for these particular species. Nevertheless, the possibility exists that different ungulate species vary in response to different feeding deterrents (Oh et al., 1967; Zahorik and Houpt, 1991). Furthermore, exposure to certain compounds can trigger learning processes (Provenza, 1996; Spalinger et al., 1997; Tixier et al., 1998) or adaptations of the rumen flora (Oh et al., 1967) with the consequence that the reaction of the animal will depend on whether it had already encountered a compound in its diet or not.

Vourc'h et al. (2001, 2002) have shown that Sitka black-tailed deer (*Odocoileus hemionus sitkensis*) prefer western redcedar (*Thuja plicata* Dann ex D. Don) leaves that have low monoterpene concentrations. Based on this, the purpose of this study was to test directly the deterrent effect of western redcedar monoterpenes on Sitka black-tailed deer, independently of the plant material the compounds are found in. To explore whether our results applied to other conifer foraging deer species, we ran a second set of experiments with European roe deer (*Capreolus capreolus*), a temperate deer species with similar body size to Sitka black-tailed deer. Finally, we tested the effect of these compounds on a tropical deer species with little dependence on conifers as a food source, the rusa deer (*Cervus timorensis russa*).

METHODS AND MATERIALS

Monoterpenes. Two oxygenated monoterpene isomers (α,β-thujone) and four hydrocarbon monoterpenes (sabinene, myrcene, α-pinene and $d + l$-limonene) have been identified by Vourc'h et al. (2001) in western redcedar leaves and were tested in feeding choice experiments. Monoterpenes were obtained from

Aldrich Chemical (Milwaukee, Wisconsin, USA) except for sabinene, which was obtained from R. C. Treatt (Bury St. Edmunds, Suffolk, England). For α,β-thujone, sabinene, and myrcene, only solutions of technical-grade purity could be obtained. The thujone solution was extracted from western redcedar leaf and contained 65% α,β-thujone (67% β-thujone and 33% α-thujone at equilibrium), 15% fenchone, 10% bornyl acetate, 3% camphor, and a mixture of other minor terpenes. The sabinene solution contained 70% sabinene, 20% β-pinene, 4% α-pinene (tested as pure solution in our experiment), and a mixture of other minor terpenes. The myrcene solution contained 75% myrcene and other minor terpenes. Although these impurities may introduce some noise in the analysis of the effect of the main compounds, they all consist of compounds that occur commonly in most terpene-producing plants and are, for the most part, characterized by low biological activity (T. P. Clausen, personal communication).

We first tested these solutions on Sitka black-tailed deer, roe deer, and rusa deer (with a simplified protocol), with the concentration corresponding, for each monoterpene, to the mean concentration found in young western redceder foliage (Vourc'h et al., 2002). Trees of this age have the highest natural monoterpene concentration observed in western redcedars (Vourc'h et al., 2001, 2002). We defined this concentration as *in vivo* redcedar concentration.

Second, to analyze the effect on monoterpene identity, we tested these solutions at different concentrations, with Sitka black-tailed deer and roe deer. On the basis of the experiment with monoterpenes at *in vivo* redcedar concentration on Sitka black-tailed deer, we separated the solutions into two groups: (1) strongly repellent, meaning deer ate food containing monoterpenes in less than 50% of the trials, and (2) weakly repellent, meaning food containing monoterpenes was eaten in more than 50% of the trials. We then tested whether the strongly repellent solutions retained activity at lower concentrations, defining lower concentration as the minimum concentration of each monoterpene found in young western redcedar foliage (Vourc'h et al., 2002). We tested the second group of solutions at higher concentrations, defining it as the concentration of the most concentrated monoterpene in redcedar foliage, i.e., α,β-thujone. We refer to these two levels as "manipulated concentrations" throughout the remainder of the paper.

The amount of monoterpene solution (MonoSol) to be used in the experiments was calculated by taking into account the concentration that we wanted to achieve (MonoConc), the purity of the manufactured monoterpene solution (MonoPurity), the density of the compound (MonoDensity), and the dry weight of the food substrate (SubstrateDW):

$$\text{MonoSol (ml)} = \frac{\dfrac{\text{MonoConc (mg/g)}}{1000} \times \text{SubstrateDW (g)}}{\text{MonoDensity (g/ml)} \times \dfrac{\text{MonoPurity (\%)}}{100}}$$

This amount was put into 5 ml solvent (85% ethanol and 15% methanol) per 100 g of fresh substrate to form the monoterpene solution. The control solution was made of 5 ml solvent per 100 g of fresh substrate.

Black-Tailed Deer on Haida Gwaii. Feeding choice experiments with Sitka black-tailed deer involved wild deer living on Haida Gwaii (Queen Charlotte Islands, British Columbia, Canada), where western redcedar is a preferred food for that species (Pojar et al., 1980; Coates et al., 1985). To our knowledge, this is the first study testing the effect of secondary metabolites on wild ungulates. Sitka black-tailed deer were introduced from the nearby mainland to Haida Gwaii near the beginning of the 20th century (Carl and Guiguet, 1972). At that time, there was no other large herbivores on the archipelago (Cowan, 1989). Five male and four female free ranging black-tailed deer of Haida Gwaii were used at three different sites: one "natural site" (Vertical Point), and two logging camps (Beattie Anchorage and Eden Lake). One female and two males were 1–2 years old; the others were mature adults. At Vertical Point, deer got used to human presence within a few days. In logging camps, deer were already used to being close to humans.

Black-tailed deer were habituated to eating apples with the solvent for a few days before proceeding to the experiments. For each trial, we prepared two bowls with 100 g of fresh cut apples each. One bowl was sprayed with a monoterpene solution and one with the control solution. We then presented the pair of bowls to one individual deer and let it eat until it moved away. Because we did not stop the trial until the deer moved away, the deer could eat off either bowl. We weighed the amount of food left in each bowl to calculate the amount of food eaten. No weight changes in the food substrate occurred because of the short duration of each trial (3–30 min). Our protocol was to run three to five trials per deer per monoterpene solution at a given concentration. The order of monoterpenes was randomized per deer for a given concentration, starting with the *in vivo* redcedar concentration. No more than three trials per deer and per day were run. The experiment lasted three weeks.

Roe Deer in France. We ran experiments with roe deer raised in captivity at the Centre d'Etudes Biologiques de Chizé (France). Roe deer eat a wide range of plant species from all major taxonomic groups including fungi, ferns, shrubs, deciduous trees, and conifers (Duncan et al., 1998). During the experiment, roe deer had access to a small forest patch with understory and a grassy area. They also had *ad libitum* access to pellets (Caprilat: 18% protein, 3% fat, 8.7% cellulose, 7.2% ash) and water.

The captive roe deer were very sensitive to human presence and could not be kept in individual pens. Therefore, we could not work with individual deer and had to run the experiments in four pens with three to five deer in each (pen 1: three females; pen 2: four females and one male; pen 3: two females and one male; and pen 4: two females and one male). Pens ranged from a 450 m^2 to 1500 m^2 pasture and 2000 m^2 forest. For a given trial in a pen, we presented as many pairs of

monoterpene and control bowls as there were deer in the pen. Each monoterpene bowl contained 100 g of apples with the same monoterpene solution and the control bowl contained 100 g of apples with solvent similar to the experiments with Sitka black-tailed deer (discussed above). We recorded the number of deer that took part in each trial as well as the number of pairs of bowls that were used. After a 2-hr trial, the total amount of control or monoterpene apples remaining in the bowls was weighed for each pen. Because the apple masses could change during the 2 hr that the trials lasted, we put a bowl with 100 g of apples outside each pen and weighed the bowl again at the end of each experiment. The amount of apple consumed was then calculated using the equation:

$$[(H_i \times O_f / O_i) - H_f]$$

where H_i and H_f were the initial and final masses of apples exposed to herbivory, and O_i and O_f the initial and final masses of apples outside the pens (Cronin and Hay, 1996). Our protocol was to run three to five trials per monoterpene solution per pen per concentration at a rate of two trials per day (one in the morning and one in the evening) in each pen. We randomized the order of monoterpene that were tested for a given pen, starting with the *in vivo* redcedar concentration. This experiment took place over a period of two months.

Rusa Deer in New Caledonia. Rusa deer were introduced to New Caledonia from the Indonesian island of Java in the 1870s (Chardonnet, 1988). Before the colonization of the island by Europeans in 1845, there were no large herbivores (Gargominy et al., 1996). Four domesticated rusa deer (a subadult male and a 1- or 2-year-old female, a 2- or 3-year-old stag, and a hind with her young) were available for experiments at the research Station of Port-Laguerre. Before the trials, the deer were raised in paddocks with cultivated grass (*Brachiaria mutica*, *Brachiaria decubems*, and *Pennisetum prurpureum*) and trees (*Calliandra calothrysus* and *Leucaena leucocephala*). During the trials, a similar diet was cut and provided daily to the animals in their cages, where they had *ad libitum* access to water.

For each trial, we presented a pair of monoterpene and control bowls to individual deer as in the experiments with Sitka black-tailed deer. The food substrate was altered to 20 g of fresh bread in each bowl. We tested monoterpenes at *in vivo* concentrations. Assays with myrcene had to be canceled because the quality of the chemical sample deteriorated. Three trials were run per day per deer using the same monoterpene solution. The order of the monoterpenes tested was randomized per deer. The experiment lasted three weeks.

Statistical Analyses. Data for each deer species were analyzed separately. For black-tailed and rusa deer, the statistic unit was a one-deer trial with a single monoterpene at a given concentration. For roe deer, the unit was a one-pen trial with one monoterpene at a given concentration. First, we calculated the frequency of zero values per monoterpene solution at each concentration, i.e., the frequency

of control or monoterpene bowls not being eaten. Then, for each trial, we calculated a log ratio of the proportion of food eaten for each option according to the method described in Elston et al. (1996):

$$\text{Log}\frac{\left(\frac{\text{g of treated food eaten}}{\text{g of treated food presented}}\right)}{\left(\frac{\text{g of control food eaten}}{\text{g of control food presented}}\right)} = \text{Log}\frac{(\text{g of treated food eaten})}{(\text{g of treated food eaten})}$$

For each trial, the sum of the proportions for each option must be 1, i.e., the data are compositional. Therefore, the log ratio transformation was the most appropriate, as it led to analyses in which the unit-sum constraint was automatically satisfied (Elston et al., 1996). If the proportion of food eaten for each option was equal, the log ratio was zero; if deer ate more control food, the log ratio was negative. To deal with the problem of 0 values, i.e., when either the control or treated food was not eaten, we replaced all the 0 values by 0.1 (Elston et al., 1996). To check the robustness of the replacement, we conducted the same analyses with values of 0.5, 1, and 2 instead of zeros, 0.5 g and 1 g being the smallest amount of bread and apple eaten, respectively. The results were consistent.

For black-tailed and roe deer, we tested the effect of concentration and monoterpene identity (= solution effect) by mixed analysis of variance of the log ratios data using the procedure MIXED in SAS (1999). The factors of interest (i.e., solution, concentration, and solution $\times$ concentration) were declared as fixed effects, whereas additional variables assumed to impact the variability of the data were declared as random effects: for black-tailed deer, deer identity and its interaction with fixed effects, as well as the rank of trial nested in deer, for roe deer, pen number and its interaction with the fixed effects, as well as the rank of trial nested in pen, the number of deer taking part in the trial in each pen, and the number of pairs of bowls from which the food was eaten at each trial. The same procedure was used for rusa deer except that the concentration effect in the mixed analysis of variance was not considered. Therefore, the fixed effect was the solution effect, and random effects were deer identity and its interactions with the solution effect, as well as the rank of the trial nested in deer. The variances of the random effects parameters became the covariance parameters. The selected structure was fitted to the data using the method of restricted maximum likelihood (REML). A likelihood-based scheme was used to infer type III F statistics to test the significance of the fixed effects (SAS, 1999). Mean differences were tested by Lsmeans (SAS, 1999). Lsmeans were also used to test whether the log ratios for each monoterpene at each concentration were significantly negative, i.e., whether the monoterpenes had a repellent effect.

RESULTS

All three species of deer smelled the food before tasting it.

Black-Tailed Deer in Haida Gwaii. In all but two of the 10 treatments, each deer was tested at least three time (Table 1). Deer tried the apples in bowls with α,β-thujone at *in vivo* redcedar concentration in only 17% of the trials. For the other monoterpenes, deer tested the bowls with monoterpene solutions in more than 50% of the trials and ate apples in 98% of the control bowls (Figure 1). The only three instances in which deer did not touch control bowls occurred in trials with α-pinene at *in vivo* redcedar concentration (i.e., in 10% of trials for this solution). The percentage of apples actually consumed in bowls with α,β-thujone was less than 2%. It varied between 40 and 70% for the other monoterpenes, whereas the percentage of apples consumed in control bowls was over 80% on average (Figure 2). Figure 3 shows a representative example of a trial for thujone. Because deer ate apples from bowls with α,β-thujone in less than 50% of the trials, α,β-thujone was tested at a diluted manipulated concentration. The other four monoterpenes solutions (sabinene, myrcene, α-pinene, and $d + l$-limonene) were all tasted by deer in more than 50% of the trials. We tested them at one elevated manipulated concentration.

TABLE 1. EFFECTS OF MONOTERPENE SOLUTIONS[a]

	Proportion of pens									
	Thujone		Sabinene		Myrcene		α-Pinene		$d + l$-Limonene	
	In vivo	Manip.	In vivo	Manip.	In vivo	Manip.	In vivo	Manip.	In vivo	Manip.
Sitka black-tailed deer [deer (N) with 3–5 trials/total deer (N) tested]	7/9 a[b]	9/9	9/9	5/9 b	9/9	9/9	9/9	9/9	9/9	9/9
Roe deer [pens (N) with 3–5 trials/total pens (N) tested]	4/5 c	2/2	4/5 c	2/2	5/5	2/2	4/5 c	2/2	5/5	2/2
Rusa deer [deer (N) with 3–5 trials/total deer (N) tested]	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4

[a] The number of trials was at least 3 and at most 5 in each test. Concentrations of compounds were at *in vivo* levels and at manipulated higher (thujone) or diluted (other compounds) levels.
[b] a, 2 deer with 1 trial only; b, 4 deer with 1 trial only; c, 1 pen with 2 trials only.

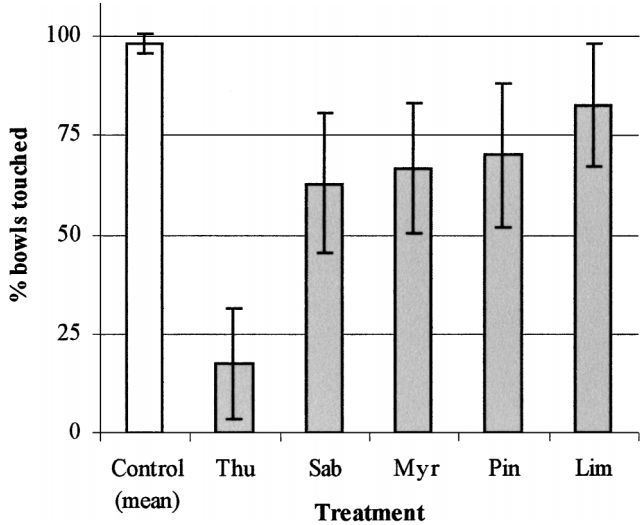


FIG. 1. Percent of bowls with apples that were touched by Sitka black-tailed deer for trials with monoterpene solutions at *in vivo* redcedar concentration or with a control solution. The percentage of control bowls touched is an average over all trials. Vertical bars are 5% confidence intervals bars. Thu = α,β-thujone, Sab = sabinene, Myr = mycrene, Pin = α-pinene, and Lim = $d + l$-limonene.

Deer tasted the content of bowls with the α,β-thujone solution at diluted concentration in 28% of the trials and consumed 12% of the apples. They tasted the content of bowls with elevated concentration of the other monoterpenes in 54–68% of trials, consuming 25% (pinene) to 35% (sabinene) of the apples (Figure 4). Deer tasted the apples in all control bowls, consuming 80–97% of the apples.

For each solution and at both concentrations, the repellent effect was significant, i.e., the mean of the log ratio was significantly smaller than zero ($P < 0.05$ for all cases). Mixed analysis of variance showed an effect of solution ($F_{4,32} = 10.07$, $P < 0.001$), concentration ($F_{1,8} = 11.43$, $P = 0.009$), and the solution $\times$ concentration interaction ($F_{4,32} = 2.99$, $P = 0.033$). The solution effect was significant at *in vivo* redcedar concentration ($F_{4,32} = 9.72$, $P < 0.001$), and at manipulated concentrations ($F_{4,32} = 3.62$, $P = 0.015$). α,β-Thujone solutions were more repellent than the other solutions at both concentrations ($P < 0.05$) (Figures 2 and 4). The repellent effect was greater at the manipulated concentration than at the *in vivo* redcedar concentration for α-pinene and for $d + l$-limonene ($P < 0.05$), but not for the other compounds.

Roe Deer in France. A total of 10 of 14 roe deer took part in the trials: all three deer in pen 1, three of five deer in pen 2, and two of three in pens 3 and 4. Deer in pens 3 and 4 were shy and only took part in the first set of experiments

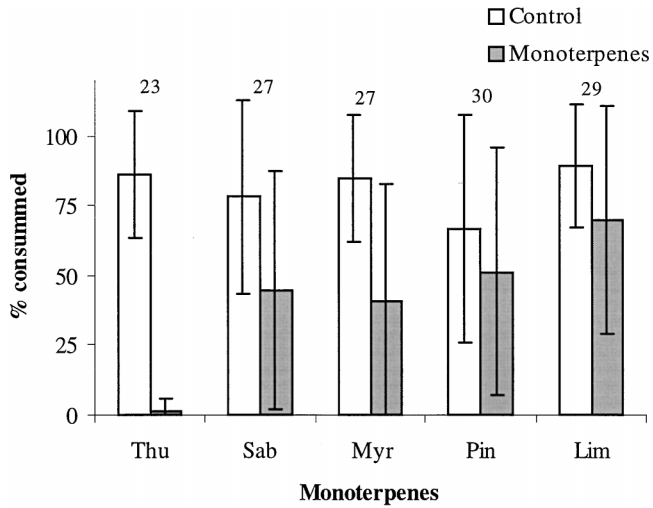


FIG. 2. Average percent of apples consumed by Sitka black-tailed deer in trials with different monoterpene solutions at *in vivo* redcedar concentration or with a control solution. Number of trials per deer varied from 3 to 5 (with some exceptions, see Table 1). Numbers above bars are number of trials. Vertical bars are standard deviations, abbreviations as in Figure 1. All differences in consumption between control and monoterpene bowls are significant ($P < 0.05$).

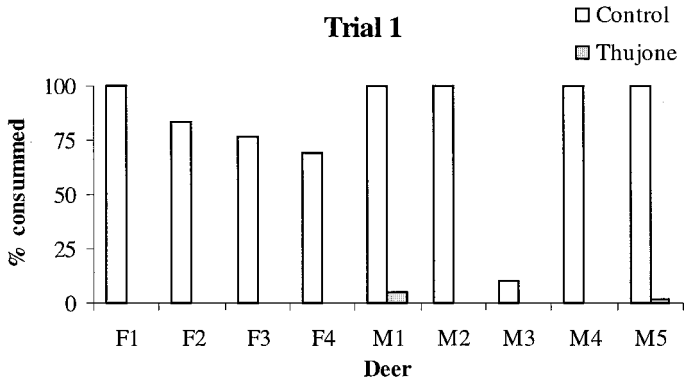


FIG. 3. Representative example of effect of α,β-thujone on Sitka black-tailed-deer consumption. Thujone = bowl of apple with α,β-thujone solution at *in vivo* redcedar concentration. Control = bowl of apple with control solution. F1 to F4 = females 1–4, M1–M5 = males 1–5.

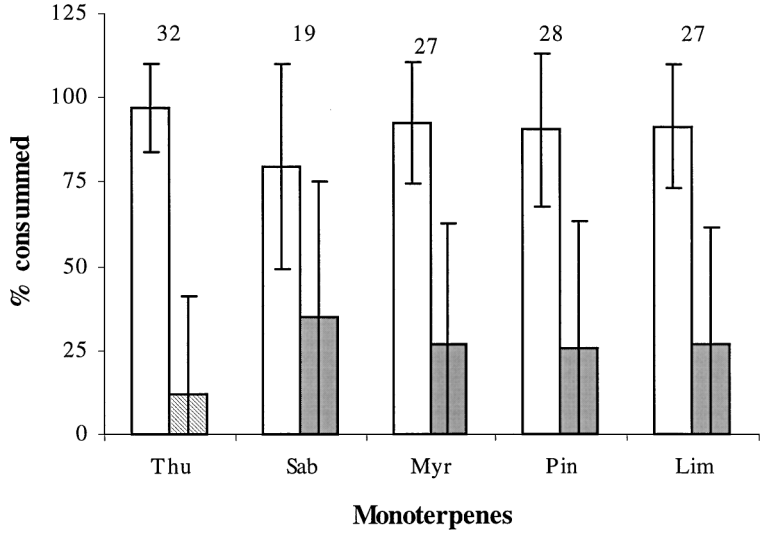


FIG. 4. Average percent of apples consumed by Sitka black-tailed deer in trials with different monoterpene solutions at manipulated concentrations. Hatched bars = monoterpene tested at lower than *in vivo* concentration; shaded bars = monoterpenes tested at higher than *in vivo* concenterations; open bars = control solutions. Vertical bars are standard deviations. Numbers above bars are number of trials. Abbreviations as in Figure 1. All differences in consumption between control and monoterpene bowls are significant ($P < 0.05$).

(*in vivo* concentrations). Except for three cases, at least three trials were run for each of the pens tested (Table 1).

Deer ate apples from the control bowl in each trial except once for the α-pinene, myrcene, and $d + l$-limonene trials at *in vivo* redcedar concentration. At *in vivo* concentrations, food with α,β-thujone was tested in 40% of the trials, that with sabinene in 94%, myrcene in 88%, α-pinene in 62%, and $d + l$-limonene in 65%. Deer tested 62% of bowls with α,β-thujone at diluted manipulated concentration. At elevated manipulated concentration, deer tested 63% of the bowls with sabinene, myrcene, and α-pinene, and 75% of the bowls with $d + l$-limonene. In the trials at *in vivo* concentrations, deer ate less than 2% of the apples with α,β-thujone solution, ate between 19 and 27% of the apples with the other monoterpene solutions, and about 50% of the apples in the control bowls (Figure 5a). In the trials at manipulated concentrations, deer ate about 13% of the apples in the bowls with α,β-thujone at diluted concentration and 9–20% of the apples in trials with the other monoterpenes at elevated concentrations (Figure 5b). In these experiments, deer ate 68–82% of the apples in the control bowls. Figure 6 shows, as an example, the results of the trials for sabinene.

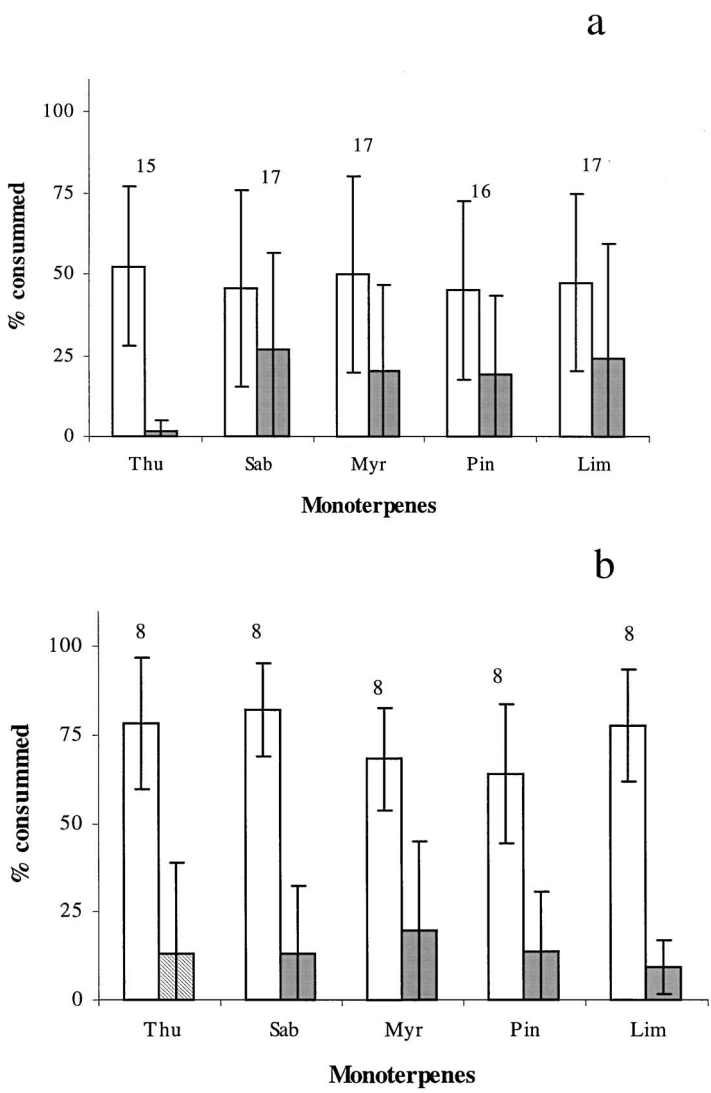


FIG. 5. Average percent of apples consumed by roe deer in trials with different monoterpene solutions at *in vivo* redcedar concentrations (a) and at manipulated concentrations (b). Vertical bars are standard deviations. Numbers above bars are number of trials pooled for all pens. (a) Shaded bares = monoterpene solutions, open bars = control solutions. (b) Hatched = monoterpenes tested at lower than *in vivo* concentration; shaded = monoterpenes tested at higher than *in vivo* concentration; open bars = control solutions. Abbreviations as in Figure 1. All differences in consumption between control and monoterpene bowls are significant ($P < 0.05$), except for sabinene at *in vivo* concentration.

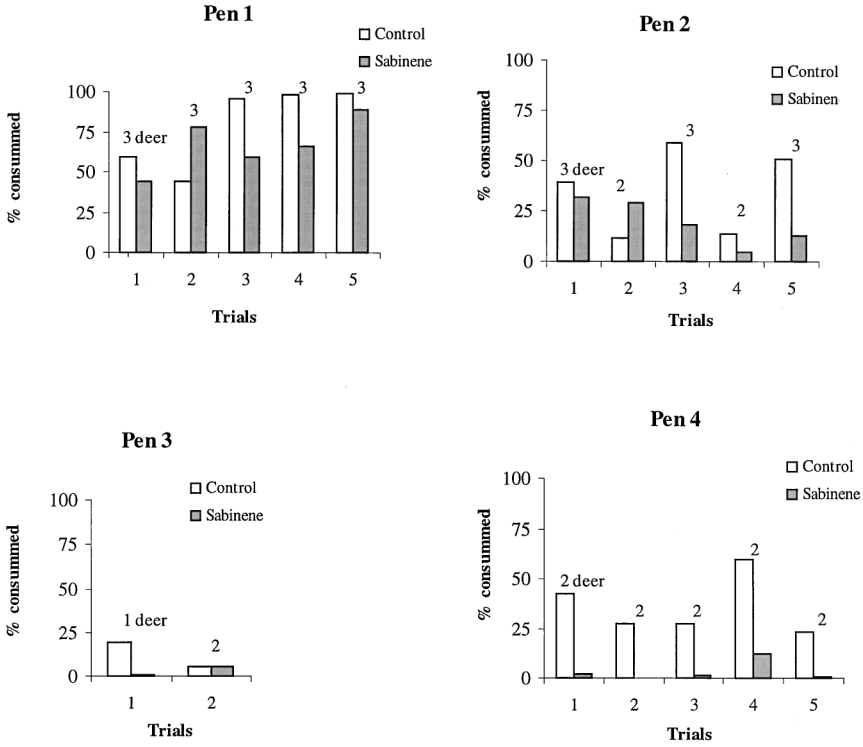


FIG. 6. Example of weak response to sabinene by roe deer from different pens. Deer had access to bowls with apples plus a control solution (control) or with apples plus a sabinene solution at *in vivo* redcedar concentration (sabinene). Numbers above bars are number of deer taking part in the trial in each pen.

The mixed analysis of variance showed no significant effect of the interaction term solution × concentration; therefore, we tested the single effects without the interaction. The solution effect was not significant ($F_{4,8} = 1.84$, $P = 0.207$), nor was the concentration effect ($F_{1,1} = 1.99$, $P = 0.393$). Even though α,β-thujone tended to be more repellent than other solutions at *in vivo* redcedar concentration (Figure 5), the solution effect was not significant either at *in vivo* ($F_{4,12} = 2.25$, $P = 0.124$) or at manipulated concentrations ($F_{4,4} = 0.05$, $P = 0.993$).

For each monoterpene at each concentration, the repellent effect was significant ($P < 0.05$ for all cases), except for sabinene at *in vivo* redcedar concentration ($P = 0.746$).

Rusa Deer in New Caledonia. Three trials were run per monoterpene solution and per deer. Of the bowls with α,β-thujone, sabinene, $d + l$-limonene, and α-pinene, 12%, 74%, 75%, and 100%, respectively, were touched. Note that myrcene

was not tested. All control bowls were touched, and over 95% of their bread was eaten. The percentage of bread eaten in bowls with the α,β-thujone solution at *in vivo* concentration was 2% and was 47%, 88%, and 57%, respectively, for sabinene, α-pinene, and $d + l$-limonene.

The mixed analysis of variance showed a significant effect of solution ($F_{3,9} = 15.05$, $P < 0.001$) at *in vivo* redcedar concentration, the only concentration used. The repellent effect was significant for α,β-thujone, sabinene, and $d + l$-limonene ($P < 0.010$ in each case), but not for α-pinene ($P = 0.315$).

DISCUSSION

Monoterpenes Are Repellent to Deer. The results of this study show that monoterpenes are feeding deterrents to deer. These results confirm the correlation observed between monoterpene content and western redcedar foliage choice by Sitka black-tailed deer (Vourc'h et al., 2001, 2002). The deterrent effect was strongest for the thujone solution, oxygenated monoterpene isomers, extracted from western redcedar leaves. This contrasted with the lower effect of the rest of the monoterpenes tested, which were all hydrocarbon monoterpenes. There was no difference between the repellent effect of these hydrocarbon monoterpenes. A stronger repellency of oxygenated monoterpenes has been suggested by Oh et al. (1967) and Schwartz et al. (1980) but has not always been confirmed (Estell et al., 2000). Diluting the concentration of α,β-thujone did not decrease its repellency. Increasing the concentration of hydrocarbon monoterpenes did increase their repellency for Sitka black-tailed deer. The same trend was observed for roe deer, but was not significant (deer from two of four pens did not take part in the trials on manipulated concentrations, reducing the power of the statistical tests).

Variation among Deer. Despite differences in the experimental protocols, we observed a remarkable homogeneity in the response pattern across species, suggesting a broad effect of monoterpenes on deer food choice. The experiments also revealed some variation in the response of the three deer species studied. At *in vivo* redcedar concentrations, sabinene and α-pinene were not repellent to roe and rusa deer, respectively, but were to Sitka black-tailed deer. A repellent effect of α-pinene on sheep was also observed by Estell et al. (1998b) and on red deer by Elliott and Loudon (1987) at concentrations similar to those in our study. At a lower concentration, however, Estell et al. (2000) did not find a deterrent effect of sabinene on sheep. Finally, Sitka black-tailed deer, which include western redceder in their regular diet, responded more strongly to α,β-thujone than roe deer, which are usually not exposed to redcedar.

Signal Effect and Consequences. Our observations of deer first smelling their food before eating or avoiding it are consistent with the findings of Elliott and London (1987) that indicate it is the odor of monoterpenes that deter red deer. However, for a plant defense to be effective, a signal has to be associated with the

negative consequences of eating the plant (Lawler et al., 1999). Indeed, if there is no physiological consequence associated with a signal, deer will ultimately learn that the food is palatable (Tixier et al., 1998; Villalba and Provenza, 2000). The nature of these negative postingestive effects of monoterpenes is still under investigation. Studies on small mammals (Millet et al., 1981; Hiroi et al., 1995; Höld et al., 2000) have suggested that it could be neurotoxicity. *In vitro* experiments by Nagy et al. (1964), Oh et al. (1967, 1968), and Schwartz et al. (1980) suggested that monoterpenes could inhibit rumen microbial fermentation, but studies based on rumen inocula (Welch and Pederson, 1981; Cluff et al., 1982) suggest that monoterpenes in the rumen might not reach concentrations high enough to affect rumen microorganisms. Further work is needed to fully understand the effect of monoterpenes on cervid digestion.

Monoterpene Effects on Foraging in the Wild. The influence of monoterpenes on deer foraging in the wild is another area where additional work is needed. Indeed, the strong repellent effect of monoterpenes we observed seems to be at odds with the regular consumption (and even the preference) of western redcedar by Sitka black-tailed deer (Pojar et al., 1980; Coates et al., 1985) and with the habit of roe deer to forage on various monoterpene-containing conifers (Duncan et al., 1998). This discrepancy could result from deer in the wild not being able to access more palatable food or from monoterpenes in conifer leaves being less detectable than monoterpenes sprayed on food substrate. It also suggests that deer can deal with monoterpenes once they are ingested, either through detoxification and/or by regulating their toxin intake according to food flavor, toxin, nutrient, and dietary experience (Provenza, 1995, 1996).

For tropical rusa deer, the context is different, as they live on New Caledonia where conifers are present, but whose contribution to deer diet is unknown. Rusa deer may encounter a much more extensive assortment of plants and could have developed a rumen flora and feeding behavior adapted to a more varied array of plant secondary metabolites including monoterpenes (Oh et al., 1967).

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SYSTEMIC, GENOTYPE-SPECIFIC INDUCTION OF TWO HERBIVORE-DETERRENT IRIDOID GLYCOSIDES IN *Plantago lanceolata* L. IN RESPONSE TO FUNGAL INFECTION BY *Diaporthe adunca* (ROB.) NIESSEL

HAMIDA B. MARAK, ARJEN BIERE,* and JOS M. M. VAN DAMME

Department of Plant Population Biology
Netherlands Institute of Ecology
NIOO-KNAW, Boterhoeksestraat 48
6666 GA Heteren, The Netherlands

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Abstract—Iridoid glycosides are a group of terpenoid secondary plant compounds known to deter generalist insect herbivores. In ribwort plantain (*Plantago lanceolata*), the iridoid glycosides aucubin and catalpol can be induced following damage by insect herbivores. In this study, we investigated whether the same compounds can be induced following infection by the fungal pathogen *Diaporthe adunca*, the causal agent of a stalk disease in *P. lanceolata*. Significant induction of aucubin and catalpol was observed in two of the three plant genotypes used in this study following inoculation with the pathogen. In one of the genotypes, induction occurred within 6 hr after inoculation, and no decay was observed within 8 days. The highest level of induction was observed in reproductive tissues (spikes and stalks) where infection took place. In these tissues, iridoid glycoside levels in infected plants were, on average, 97% and 37% higher than the constitutive levels in the corresponding control plants, respectively. Significant induction was also observed in leaves (24%) and roots (17%). In addition to significant genotypic variation in the level of induction, we found genetic variation for the tissue-specific pattern of induction, further broadening the scope for evolutionary fine-tuning of induced responses. Recent studies have revealed a negative association between iridoid glycoside levels in *P. lanceolata* genotypes and the amount of growth and reproduction of *D. adunca* that these genotypes support. However, for the three genotypes used in the present study, differences in resistance were not related to their constitutive or induced levels of iridoid glycosides, suggesting that additional resistance mechanisms are important in this host-pathogen system. We conclude that iridoid glycosides in *P. lanceolata* can be induced both by arthropods and pathogenic micro-organisms. Pathogen

* To whom correspondence should be addressed. E-mail: biere@cto.nioo.knaw.nl

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infection could, therefore, potentially enhance resistance to generalist insect herbivores in this species.

Key Words—Constitutive chemical defense, induced chemical defense, *Diaporthe adunca*, genetic variation, *Plantago lanceolata*, plant–pathogen interactions, plant–herbivore interactions.

INTRODUCTION

The role of secondary plant compounds in defense against pathogens has been studied intensively during the past decades (e.g., Bailey and Mansfield, 1982; Harborne, 1993; Hammerschmidt and Kuc, 1995). A wide variety of secondary metabolites appears to be involved in pathogen resistance. Whereas some of these compounds are produced constitutively, many others are only produced in response to pathogen infection, either by hydrolysis or oxidation of preexisting nontoxic substrates, or *de novo* by gene derepression or activation of enzyme systems, as in the case of phytoalexins (Harborne, 1993). An aspect of secondary plant metabolites that has recently attracted attention is their specificity, both with respect to the types of organisms that can induce them and with respect to the types of organisms that they can affect (e.g., Krischik et al., 1991; Barbosa, 1991; Harborne, 1993; Hammerschmidt and Schultz, 1996; Karban and Baldwin, 1997; Paul et al., 1999; Felton and Korth, 2000; Stout and Bostock, 2000). For instance, phytoalexins are generally considered to be specifically active against pathogenic microorganisms, but they often appear to affect the performance of insect herbivores as well. In fact, several groups of secondary plant compounds have been shown to play a role in defense against both insect herbivores and pathogens (for reviews see, e.g., Krischick et al., 1991; Harborne, 1993; Hatcher, 1995; Hammerschmidt and Schultz, 1996), which has raised the question of whether secondary metabolites could serve a "generalized defense function" (Krischik et al., 1991). Groups that can function both as antiherbivory and antimicrobial compounds include isoflavonoids in legumes (Kogan and Fischer, 1991), inducible terpenes and phenolics in conifers (Klepzig et al., 1996), and many alkaloids (Hartmann, 1991) such as nicotin in tobacco (Krischick, 1991), glycoalkaloids in potato and tomato (Sinden et al., 1973, 1978; Roddick, 1974), and quinolizidine alkaloids in lupins (Wink, 1984). Many of these compounds can be induced by either or both types of biotic agents, and could, thus, mediate induced cross-resistance between pathogens and insect herbivores (Hammerschmidt and Schultz, 1996).

An interesting group of molecules in this respect are the iridoid glycosides. Iridoid glycosides are a group of monoterpene derivates found in a large number of plant families (Jensen, 1991) that are produced via the isoprenoid biosynthetic pathway (McGarvey and Croteau, 1995). Their biological effects on insect herbivores are well documented. They generally deter feeding by generalist insect

herbivores but can be used as feeding or oviposition stimulants by some specialists (Bowers, 1991). For instance, the iridoid glycosides aucubin and catalpol that occur in ribwort plantain (*Plantago lanceolata*) reduce the growth of the generalist herbivore *Lymantria dispar*, but they enhance growth of the specialist *Junonia coenia* (Bowers and Puttick, 1988) that uses these compounds as feeding and oviposition stimulants (Pereyra and Bowers, 1988; Bowers and Puttick, 1989; Klockars et al., 1993) and sequesters them for its own defense against predators (e.g., Dyer and Bowers, 1996; Bowers and Stamp, 1997; Camara, 1997). Biological effects of iridoid glycosides on bacterial and fungal microorganisms have also been documented. *In vitro* studies have shown antimicrobial activity of the aglycon form of aucubin and catalpol (the products of enzymatic conversion by β-glucosidase enzymes) (Rombouts and Links, 1956; Ishiguro et al., 1982; Van der Sluis et al., 1983; Davini et al., 1986; Stermitz, 1988). In accordance with these studies, we have recently shown *in vitro* inhibiting effects of the aglycon form of aucubin and catalpol on the growth of *Diaporthe adunca*, a specialist phytopathogenic fungus of *P. lanceolata* (Marak et al., 2002). In addition, our studies suggest *in vivo* antimicrobial effects of these compounds; growth and reproduction of *D. adunca* were significantly reduced on *P. lanceolata* plants artificially selected for high constitutive levels of iridoid glycosides compared to plants selected for low levels of these compounds, the latter group having on average approximately fourfold lower leaf levels of aucubin and catalpol (Marak et al., 2000). Insect herbivores can induce aucubin and catalpol in *P. lanceolata* (Bowers and Stamp, 1993; Darrow and Bowers, 1999; but see Jarzomski et al., 2000), but whether infection by pathogenic fungi can also result in their induction, and hence in a potential increase in resistance to generalist insect herbivores, is as yet unknown.

In this paper, we investigate whether aucubin and catalpol, the main iridoid glycosides that occur in *P. lanceolata*, can be induced following infection with the specialist fungus *D. adunca*. In addition, we investigate the phenological pattern of induction, whether induction is local or systemic, whether it is tissue-specific, and whether the level of induction differs among plant genotypes within a population. Genetic variation for the level of induction of chemical defense has been documented for several natural plant–herbivore systems, but evidence from natural plant–pathogen systems is scarce (Agrawal, 2000). Such variation is a prerequisite for any evolutionary response to selection for increased or decreased inducibility of defense to occur. The scope for evolutionary fine-tuning of induced responses to biotic challenge would even be broader if, in addition, there is genetic variation for the tissue-specific pattern of induction (Karban and Baldwin, 1997). We address four questions: (1) Is there genetic and tissue-specific variation in the constitutive level of the iridoid glycosides aucubin and catalpol among plant genotypes? (2) Is there local and/or systemic induction of iridoid glycosides following fungal infection? (3) Does the level of induction of aucubin and catalpol differ among plant

genotypes? (4) Does the level of induction differ among plant tissues and are such allocation patterns genotype-specific?

METHODS AND MATERIALS

Host Plant. Ribwort plantain (*Plantago lanceolata* L.) is a perennial plant species with a worldwide distribution and a large ecological amplitude (Sagar and Harper, 1964). The species is typically found in roadsides, hayfields, and dry grassland (Haeck, 1992). The plant forms a rosette that can survive during the winter. Flowers are produced in spikes on elongated stalks.

Iridoid glycosides in P. lanceolata. P. lanceolata contains, among others, the two iridoid glycosides aucubin and catalpol (Duff et al., 1965). Aucubin is the biosynthetic precursor of catalpol (Damtoft et al., 1983). In natural populations, iridoid glycoside levels in *P. lanceolata* range from undetectable to ca. 9% of its dry weight (Bowers, 1991). Variation in constitutive iridoid glycoside content in *P. lanceolata* is partially genetically determined (Bowers et al., 1992; Bowers and Stamp, 1992, 1993; Adler et al., 1995). In addition, iridoid glycoside levels in *P. lanceolata* are strongly affected by environment and ontogeny. Average levels vary among habitats and populations (Darrow and Bowers, 1997) and are generally higher in plants grown under high light and low nutrient or water conditions (Fajer et al., 1992; Darrow and Bowers, 1999). Levels generally decrease with plant and leaf age, and change during the growing season (Bowers et al., 1992; Bowers and Stamp, 1992, 1993; Stamp and Bowers, 1994; Adler et al., 1995; Darrow and Bowers, 1997, 1999). Biological effects and induction of iridoid glycosides in *P. lanceolata* have been summarized in the introduction.

Pathogen. The stalk pathogen *Diaporthe adunca* (Rob.) Niessel is an ascomycetous phytopathogenic fungus. It is specialized on *P. lanceolata* and a few other species within the same plant genus (De Nooij and Van der Aa, 1987), and reduces seed production of the plant. In spring, mature asexual spores, derived from asexual fruiting bodies (pycnidia) on overwintered scapes that were infected during the previous season, infect new spikes mainly through rain splashes (Linders et al., 1996). The fungus usually attacks the host stalks (just below the spikes) and grows down the stalks, transforming the green stalks into a black, stromatic tissue with asexual fruiting bodies (pycnidia) that start to sporulate under wet conditions, producing asexual spores (conidiospores). During rain events, new healthy spikes get infected by the asexual spores from these pycnidia through horizontal splash dispersal. Infected stalks show a characteristic downward curvature and suffer reduced seed set because transport of water and nutrient to these stalks is blocked. When the fungus reaches the rosette, the rosette will blacken, and the host will eventually die. The sexual stage of the fungus is heterothallic (Linders, 1996). Perithecia (ascomata) are formed within diseased stalk tissues.

After asexual reproduction from detached, overwintered scapes in spring, perithecia develop from the same diseased stalks (from June until September), producing sexual spores (ascospores).

Cloning of Host Plants. Three plant genotypes of *P. lanceolata* were collected from a roadside population at Papendal (52°00′ N, 5°49′ E), The Netherlands. We refer to these plant genotypes as P1, P2, and P3. Each genotype was cloned into ca. 150 individual rosettes by using a root cutting method (Van der Toorn et al., 1984). The mother plants were grown in pots with a perforated bottom, and these pots were placed on top of other pots filled with compost soil. After 20 weeks, the mother plant was removed and root cuttings established in the lower pots were allowed to grow and form new rosettes for a few weeks.

Preparation of Spore Suspension. Pathogen isolates originated from a nearby roadside population of *P. lanceolata* at Wolfheze (52°00′ N, 5°48′ E), The Netherlands, in 1990. Isolates were stored in the form of experimentally infected stalks at −20°C. A single isolate (016-9002), shown to be highly virulent on a large number of plant genotypes from a number of host populations (E. G. A. Linders, unpublished data), was used for inoculation. To obtain a spore suspension for inoculation, diseased stalks (2-cm pieces) were put on potato dextrose agar (PDA) medium at 22°C. After 4 days, small disks of fungal mycelium were transferred to oatmeal agar Petri dishes containing sterilized healthy plant stalks. The fungus was grown under UV light at 22°C for 3 weeks to induce spore production. A spore suspension of asexual spores containing 10^6 conidia/ml was prepared in sterile water.

Inoculations for Testing the Level of Induction of Iridoid Glycosides. Sixty rosettes per genotype were selected and transplanted in plastic pots ($9 \times 9 \times 10$ cm) with compost–sand (4:1) soil. Pots were completely randomized and plants were grown in the greenhouse under 16 hr light at 22°C/18°C day/night. After the start of male flowering, two spikes of 30 plants per genotype were inoculated by placing 20 μl of spore suspension at the base of the spike. The inoculated spikes were kept wet for two days by covering them with moist plastic bags. Moist plastic bags also covered the spikes of the control plants (30 plants per genotype) for two days. Five replicates of both inoculated and control plants were destructively harvested 6 times after inoculation (6, 12, and 24 hr, 2, 4, and 8 days). The latent period of the fungus is usually in the order of 1–3 weeks. In accordance, no disease symptoms were observed within the duration of the experiment. Each plant was divided into root, leaf, stalk, and spike tissue. The plant material was dried in a freeze drier and ground to fine powder in a mortar.

Inoculations for Testing the Susceptibility of Host Genotypes. In a second experiment, the three genotypes were tested for resistance to *D. adunca*. Twenty-three rosettes per genotype were selected and transplanted in plastic pots ($11 \times 11 \times 12$ cm) with compost–sand (4:1) soil. Pots were randomized and plants were grown in the greenhouse under 16 hr light at 22°C/18°C day/night. After the

start of male flowering, two spikes per plants per genotype were inoculated by placing 20 μl of spore suspension at the base of the spike. The inoculated spikes were kept wet for 2 days by covering them with moist plastic bags. Starting 1 week after inoculation, lesion size (length of blackened area) of each stalk was measured weekly during 4 weeks. To determine the density of the pycnidia that were produced on the diseased stalks, the degree of blackness was scored as: 0 = no pycnidia, 1 = <50% black, 2 = >50% black, and 3 = all pycnidia fused. To assess effects of fungal infection on seed set of the three plant genotypes, spike length and the number of seeds per spike was determined both for noninoculated and for inoculated spikes of five plants per genotype.

Chemical Analyses. Iridoid glycoside content in the plant tissues was analyzed with a gas chromatograph (Gardner and Stermitz, 1988). From each plant, 25 mg of ground material from roots and leaves, 15 mg of stalks, and 10 mg of spikes were extracted in 10 ml MeOH (95%) overnight. Crude extracts were filtered (Whatman No. 4) and the filtrate was evaporated to dryness. The dry residue was dissolved in 2 ml of distilled H_2O and washed three times with 1 ml of diethyl ether. After adding 1 ml of phenyl-β-D-glucose (0.5 mg/ml) as an internal standard, the aqueous layer was evaporated to dryness and the residue was dissolved in 1 ml of MeOH. A 0.10-ml aliquot was removed and placed into a small glass tube and evaporated to dryness. A silylation reagent (Tri-Sil Z, Pierce Chemical Company) was added (0.10 ml), and the closed tube was heated at 70–80°C for 15 min. After the solution was cooled, 1 μl was injected into a gas chromatograph (HRGC Mega 2, FISONS Instruments, Roadano, Italy). The GC was equipped with a split/splitless injector, a WCCT Fused Silica capillary column (Chrompack), and an FID detector. The injector was used in the split mode with a split ratio of 30:1 and a temperature of 275°C. Carrier gas was high-grade helium (99.996%) with a column heated pressure of 12 psi and a linear velocity of 30 cm/sec (1.44 ml/min). The detector temperature was 325°C, with a hydrogen flow of 38 ml/min, air flow of 200 ml/min, and helium flow of 40 ml/min. Samples were run under the following program: initial column temperature 200°C for 1 min, which was then raised to 260°C at a rate of 20°/min and held for 8 min. Peaknet Software Release 3.1 (DX-LAN module, Dionex Corporation, Sunnyvale, California, USA) was used for peak integration and data collection. Retention time (RT) was 7.28 min and 8.29 min for aucubin and catalpol, respectively.

Data Analysis. Effects of plant genotype, tissue, harvest time, and treatment (control vs inoculation) on iridoid glycoside content were tested by a repeated-measurements ANOVA (SAS 6.12, Procedure GLM, SAS Institute, Cary, North Carolina, USA). All factors were specified as fixed effects. The experimental units in this study were pots with individual plants. Measurements of iridoid glycoside levels of the four different tissues on each individual plant can, therefore, not be considered as truly independent. To account for the resulting covariance structure, tissue was treated as a repeated effect. Hence, tissue and all interactions

with tissue were analyzed as within-subject effects. All other effects were treated as between-subject effects. We will refer to the sum of aucubin and catalpol as "total iridoids." Separate analyses were performed for aucubin, catalpol, total iridoids, and the ratio of catalpol to the total iridoids. To improve normality, all chemical data were ln-transformed prior to analysis. To facilitate a discussion of effects of tissue, genotype, and time on the constitutive levels of iridoid glycosides, a second set of analyses was carried out using data of control plants only.

Calculations of fungal growth rate (millimeters per day) on the diseased stalks were based on quadratic regressions of the length of the black, stromatic part of stalks on time. Latent period (t_0) was estimated as the intercept with the x-axis. Average growth rate of the fungus was calculated over the first 21 days after the start of the first symptoms (t_0), the maximum period covered by all plants. For each time interval, the blackness of the infected stalk tissue (pycnidia-density class) produced during that interval was scored, and a mean value weighed by the length of the stromatic part at the corresponding time interval was calculated for each stalk. Analyses of variance were carried out to test differences in fungal growth rate, latent period (ln-transformed), and blackness among the three plant genotypes (Procedure GLM, SAS Institute) followed by *a posteriori* (SNK) contrasts. Chi-square tests were used to test differences in percentage of successful infections among plant genotypes.

RESULTS

Constitutive Levels of Iridoid Glycosides. The constitutive level of iridoid glycosides differed significantly among plant genotypes and plant tissues (Table 1), but not among harvests (Table 1, Figure 1). Reproductive tissues (stalks and spikes) had significantly higher levels of iridoid glycosides than vegetative tissues (leaves and roots), and within reproductive tissues, spikes had higher iridoid glycoside levels than stalks (Table 1, orthogonal contrasts for tissue; Table 2, data for control plants). The ratio of catalpol to the total iridoid glycosides showed a similar pattern, its average value decreasing from 0.68 in spikes to 0.33 in vegetative tissues (Table 2, data for control plants). There was a significant plant genotype × tissue interaction (Table 1), indicating that the magnitude of differences in constitutive levels of iridoid glycosides among plant genotypes depended on plant tissue. Notably, genotype P1 had significantly higher constitutive levels of iridoid glycosides in reproductive tissues, but not in leaves compared to the genotypes P2 and P3 (Figure 2, control plants). Genotypes also showed significant, tissue-specific, differences in the ratio of catalpol to the total iridoid glycosides (Table 1). Genotype P2 on average had a lower catalpol-to-total iridoid ratio than the other two genotypes (Figure 3, control plants).

TABLE 1. ANALYSIS OF VARIANCE FOR EFFECTS OF PLANT GENOTYPE (GEN), HARVEST TIME (HAR), AND PLANT TISSUE (TIS) ON CONSTITUTIVE LEVELS (% OF DRY WEIGHT) OF AUCUBIN, CATALPOL, TOTAL IRIDOID GLYCOSIDES (AUCUBIN + CATALPOL), AND RATIO OF CATALPOL TO TOTAL IRIDOID GLYCOSIDES IN CONTROL PLANTS OF *P. lanceolata*[a]

		Total iridoids		Aucubin		Catalpol		Catalpol : total	
Source	*df*	MS	*F*	MS	*F*	MS	*F*	MS	*F*
Between subjects									
Gen	2	10.51	20.0***	8.12	12.7***	15.18	30.2***	21.01	23.8***
Har	5	0.97	1.9	1.30	2.0	1.02	2.0	3.53	4.0**
Gen × Har	10	1.24	2.4*	1.28	2.0*	1.13	2.2*	1.10	1.2
Error (between)	72	0.53		0.64		0.50		0.88	
Within subjects									
Tis	3	4.27	20.5***	3.26	12.7***	28.89	114.9***	243.27	380.3***
Rep. vs. veg.	1	11.51	39.7***	1.83	5.2*	71.41	190.8***	499.88	696.6***
Stalk vs. spike	1	1.05	4.4*	7.73	26.2***	14.92	55.0***	229.86	278.9***
Leaf vs. root	1	0.25	2.6	0.21	1.7	0.32	3.0	0.06	0.2
Tis × Gen	6	3.44	16.5***	3.01	11.7***	3.42	13.6***	4.06	6.4***
Tis × Har	15	1.13	5.4***	1.03	4.0***	1.46	5.8***	1.62	2.5**
Tis × Gen × Har	30	0.52	2.5***	0.47	1.8**	0.72	2.9***	1.06	1.7*
Error (within)	216	0.21		0.26		0.25		0.64	

[a] Tissue is treated as within-subjects factor (repeated measures analysis) and orthogonal contrasts for this factor are given: (1) reproductive (stalk + spike) vs. vegetative (leaf + root) tissue; (2) stalk vs. spike tissue; (3) leaf vs. root tissue. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Induction of Iridoid Glycosides as a Response to Fungal Infection. Overall, the levels of aucubin and catalpol significantly increased as a response to fungal infection (Table 3, Figures 1 and 2). The earliest observed induction (in the spikes of genotype P2, Figure 1) occurred within 6 hr after inoculation, and the average level of induction of iridoid glycosides was not significantly altered during the first week after inoculation (Table 3, no treatment × harvest interaction). The level of induction was slightly lower for catalpol than for aucubin (Table 2). Although this resulted in a significant reduction in the ratio of catalpol to the total level of iridoid glycosides (Table 3), the overall magnitude of the decrease in this ratio was rather small (Table 2, Figure 3). There were large differences in the level of induction among plant tissues (Table 3, interaction between treatment and plant tissue). The average level of induction was higher in spikes, where infection took place, and stalks (on average 97% and 37% higher than controls, respectively), than in leaves and roots (on average 24 and 17% higher than controls, Table 2). Within reproductive tissues, the level of induction was higher in spikes than in stalks (Table 3, orthogonal contrasts for the interaction between tissue and treatment). The level of induction also varied among genotypes in a tissue-specific way (Table 3). Genotype P1, which contained the highest constitutive levels of iridoid glycosides in reproductive tissues, did not show significant induction in any of the tissues

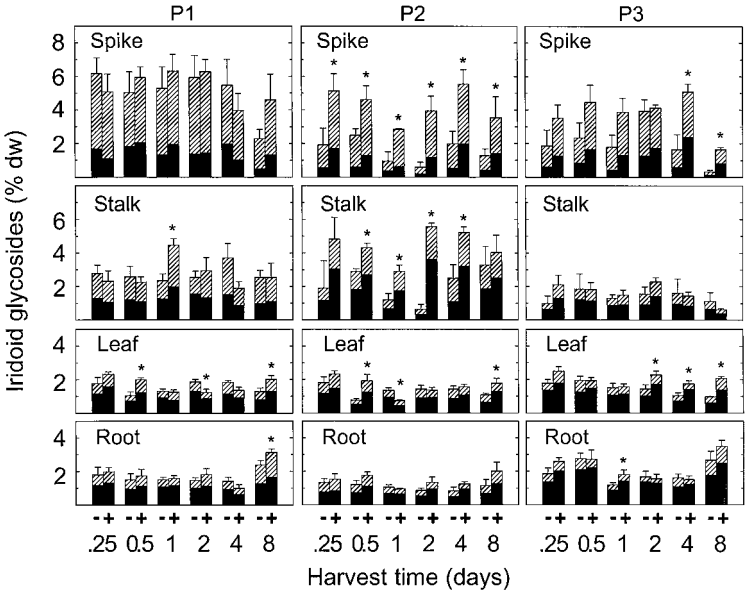


FIG. 1. Time course of the concentrations (% of tissue dry weight) of aucubin (black) and catalpol (shaded) in control (−) and inoculated (+) plants of *P. lanceolata*, between 6 hr and 8 days after inoculation. Data are for three plant genotypes, P1, P2, and P3, and four tissue types. Standard errors refer to the total iridoid glycoside (aucubin + catalpol) content. Asterisks refer to significant differences between iridoid glycoside levels in inoculated plants and their corresponding controls ($P < 0.05$).

(Figure 2). By contrast, genotypes P2 and P3, which showed low constitutive levels in reproductive tissues, showed significant induction in spikes and various other tissues (Figure 2).

Resistance to the Fungus D. adunca. The three genotypes P1, P2, and P3 differed significantly in their resistance to the pathogen isolate used in this study (Table 4). Genotype P3 had the lowest percentage of infection. Plants of this genotype that did become infected supported only limited growth and reproduction of the fungus compared to the other two genotypes. This was caused by a significantly (>1 week) longer latent period of the fungus on this genotype, as well as by a significantly lower density of asexual fruiting bodies of the fungus on infected parts of the stalks. As a result, P3 was the only genotype that was able to maintain seed production after inoculation (Table 4). Genotypes P1 and P2 were completely susceptible to the fungal isolate, and the only difference between these two genotypes was the higher density of fungal fruiting bodies produced on stalks of genotype P1. No differences in growth rate of the fungus were observed among the three plant genotypes. There was no indication that higher levels of resistance among

TABLE 2. BACK-TRANSFORMED MEANS (± SE) FOR CONCENTRATIONS (% DW) OF AUCUBIN, CATALPOL, TOTAL IRIDOID GLYCOSIDES (AUCUBIN + CATALPOL), AND RATIO OF CATALPOL TO TOTAL IRIDOID GLYCOSIDES, AVERAGED OVER GENOTYPES AND HARVEST TIMES ($N = 90$) FOR FOUR DIFFERENT TISSUES[a]

	Roots	Leaves	Stalks	Spikes
Aucubin				
Control	0.98 (0.05) b	0.92 (0.04) b	1.01 (0.08) c	0.67 (0.07) a
Infected	1.20 (0.06) a	1.16 (0.04) a	1.45 (0.01) b	1.38 (0.07) ab
% Induction	22.3**	26.2***	43.5***	106.8***
Catalpol				
Control	0.48 (0.03) a	0.44 (0.02) a	0.85 (0.07) b	1.15 (0.15) c
Infected	0.51 (0.03) a	0.53 (0.03) a	1.11 (0.08) b	2.89 (0.14) c
% Induction	4.9	19.1*	31.3*	92.0***
Total				
Control	1.50 (0.08) a	1.39 (0.06) a	1.91 (0.16) b	2.23 (0.25) c
Infected	1.76 (0.09) a	1.72 (0.07) a	2.61 (0.19) b	4.38 (0.22) c
% Induction	17.4*	23.5***	36.5**	96.7***
Catalpol:total				
Control	0.337 (0.010) a	0.334 (0.009) a	0.457 (0.012) b	0.683 (0.010) c
Infected	0.308 (0.012) a	0.321 (0.009) a	0.435 (0.010) b	0.668 (0.011) c
% Change	−8.8	−3.9	−4.9	−2.2

[a] Values within rows (across tissues) that do not share a common letter are significantly different from each other (SNK multiple comparison of means, $P < 0.05$). The level of induction (%) is expressed as [100 × (inoculated − control)/control], and the significance of induction is indicated by asterisks (one-way ANOVA of control vs. inoculated; NS is not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

the three genotypes were associated with higher constitutive or induced levels of iridoid glycosides in their reproductive tissues (Figure 2). Both the constitutive level and the total level of iridoid glycosides following infection were actually lower in the resistant genotype P3 than in the susceptible genotype P1, despite the higher level of induction in the spikes of the former genotype. Constitutive levels of iridoid glycosides were equally low in the resistant genotype P3 and the susceptible genotype P2, whereas inducible levels in the stalk were higher in the latter genotype.

DISCUSSION

Induction of Iridoid Glycosides: A Generalized Response to Various Biotic Agents. Induction of secondary plant compounds as a result of fungal, bacterial, or viral infection and the role of these compounds in resistance have been shown in a large number of plant species (e.g., Harborne, 1993; Hammerschmidt and Kuc, 1995). Overall, infection of *P. lanceolata* by the common fungal stalk pathogen

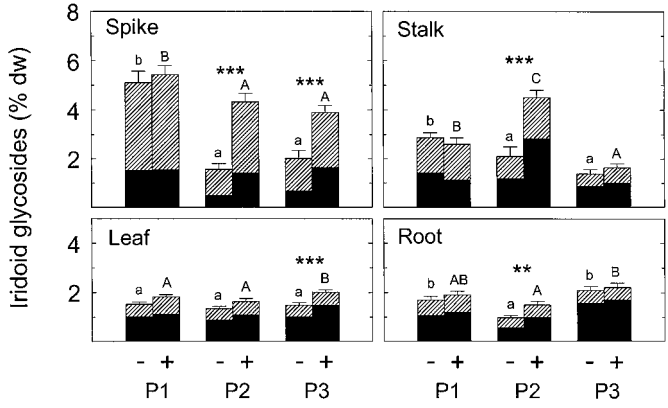


FIG. 2. Concentrations (% of tissue dry weight) of aucubin (black) and catalpol (shaded) in control (−) and inoculated (+) plants of three *P. lanceolata* genotypes, P1, P2, and P3, and four tissue types, averaged over six harvests ($N = 30$). Standard errors refer to the total levels of iridoid glycosides (aucubin + catalpol). Within each graph, total iridoid glycoside levels of genotypes that do not share a common small letter are significantly different from each other within the control treatment (SNK multiple comparison of means, $P < 0.05$). Likewise, capital letters refer to genotype differences within the inoculation treatment. Asterisks refer to significance of differences between inoculated and corresponding control plants, $^{**}P < 0.01$; $^{***}P < 0.001$.

D. adunca resulted in a significant, systemic increase in the levels of aucubin and catalpol in all above-ground plant tissues. The earliest observed induction was rapid, within 6 hr, and no decay of the response was observed within 8 days. To our knowledge, this is the first report of induction of these compounds by a pathogenic microorganism. The group of biotic agents that can induce iridoid glycosides in *P. lanceolata* is, therefore, not restricted to insect herbivores (Darrow and Bowers, 1999), but includes such disparate groups as arthropods and fungal pathogens. There is much recent interest in the degree of specificity of induced responses to arthropods and pathogens. Plants appear to possess a greater ability to distinguish between different types of biotic challenges (i.e., arthropods and pathogens) and to produce different sets of chemicals or gene products in response to each type of challenge than previously acknowledged ("specificity of response," Stout and Bostock, 2000). A growing number of studies present evidence that at least some of the many changes in the level or activity of chemical compounds, enzymes, and defense-related proteins that occur after challenge by phytophages are specific to the type of inducer. Nonetheless, concurrent changes in other compounds may be independent of the type of inducer, i.e., represent a common response to arthropod and pathogen challenge. In *P. lanceolata*, induction of iridoid glycosides appears to be part of such a common response. Growth chamber studies by Darrow and

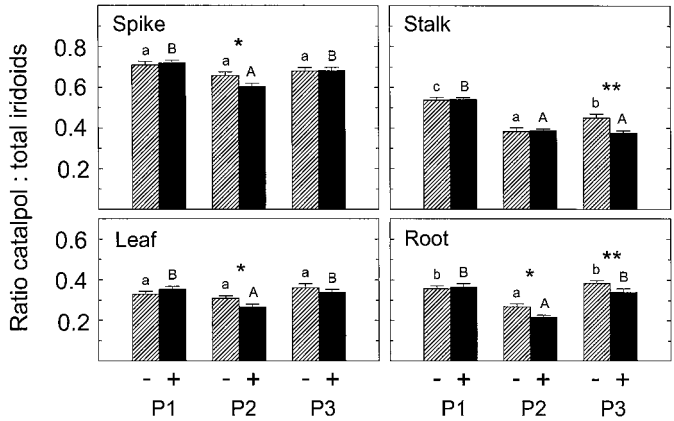


FIG. 3. Ratio of catalpol to the total level of iridoid glycosides (aucubin + catalpol) in control (−, hatched bars) and inoculated (+, black bars) plants of three *P. lanceolata* genotypes, P1, P2, and P3, and four tissue types, averaged over six harvests ($N = 30$). Within each graph, genotypes that do not share a common small letter are significantly different from each other within the control treatment (SNK multiple comparison of means, $P < 0.05$). Likewise, capital letters refer to genotype differences within the inoculation treatment. Asterisks refer to significance of differences between inoculated and corresponding control plants, $^{*}P < 0.05$; $^{**}P < 0.01$. Bars represent 1 SE.

Bowers (1999) have shown that leaf herbivory by caterpillars of the specialist insect herbivore *Junonia coenia* causes a significant increase in the levels of aucubin and catalpol in *P. lanceolata*, although experimental field studies using the same specialist herbivore have yielded more variable results. Levels of catalpol were found to increase (Bowers and Stamp, 1993), decrease (Jarzomski et al., 2000) or be unaffected (Stamp and Bowers, 1996, 2000) by exposure to *J. coenia*, whereas levels of aucubin were not affected in any of these studies. As discussed by Stamp and Bowers (1996, 2000), the inability to detect induction of iridoid glycosides by herbivory in experimental field studies may simply reflect the fact that prevailing biotic or abiotic field conditions such as summer drought can result in an induced status of both plants that are assigned to a herbivory treatment and "control" plants, hence the potential for a further increase in the level of iridoid glycosides by herbivory may be limited under such conditions. In addition to induction by insect herbivores and the fungal pathogen, studies by Gange and West (1994) suggest that increases in the level of iridoid glycosides may also result from infection by arbuscular mycorrhizae, although this should probably be viewed as an incidental consequence of an altered C/N ratio rather than as an active defense response (cf. Karban and Baldwin, 1997). Gange and West observed that treatment of *P. lanceolata* with the fungicide iprodione resulted in reduced levels of mycorrhizal

TABLE 3. ANALYSIS OF VARIANCE FOR EFFECTS OF TREATMENT (INOCULATED VS. CONTROL, TRE), HARVEST TIME (HAR), PLANT GENOTYPE (GEN), AND PLANT TISSUE (TIS) ON CONCENTRATION (% DW) OF AUCUBIN, CATALPOL, TOTAL IRIDOID GLYCOSIDES (AUCUBIN + CATALPOL) AND RATIO OF CATALPOL TO TOTAL IRIDOID GLYCOSIDES IN *P. lanceolata*[a]

		Total iridoids		Aucubin		Catalpol		Catalpol : total	
Source	*df*	MS	*F*	MS	*F*	MS	*F*	MS	*F*
Between subjects									
Tre	1	20.78	58.0***	26.06	58.4***	14.80	39.7***	7.05	8.8**
Gen	2	6.63	18.5***	2.40	5.4**	19.30	51.8***	64.32	80.5***
Har	5	1.39	3.9**	1.64	3.7**	1.47	3.9**	1.80	2.3
Tre × Gen	2	4.66	13.0***	6.25	14.0***	3.02	8.1***	4.32	5.4***
Tre × Har	5	0.35	1.0	0.47	1.1	0.74	2.0	3.40	4.3**
Gen × Har	10	1.34	3.8***	1.40	3.1**	1.13	3.0**	1.42	1.8
Tre × Gen × Har	10	0.64	1.8	0.92	2.1*	0.55	1.5	0.96	1.2
Error between	144	0.36		0.45		0.37		0.80	
Within subjects									
Tis	3	19.14	124.6***	1.72	9.0***	85.82	435.3***	495.26	931.6***
Tis × Tre	3	2.44	15.9***	2.61	13.7***	3.05	15.5***	0.25	0.5
Rep. vs. Veg.	1	4.26	20.6***	4.80	18.2***	5.54	20.5***	0.03	0.1
Stalk vs. Spike	1	3.01	19.4***	3.01	15.0***	3.26	17.6***	0.12	0.2
Leaf vs. Root	1	0.06	0.6	0.02	0.2	0.04	0.3	0.59	1.7
Tis × Gen	6	5.70	37.1***	5.78	30.2***	4.22	21.4***	7.27	13.7***
Tis × Har	15	1.30	8.45***	1.03	5.4***	2.12	10.8***	2.66	5.0***
Tis × Tre × Gen	6	1.21	7.9***	1.49	7.8***	1.14	5.8***	1.25	2.4*
Tis × Tre × Har	15	0.66	4.3***	0.71	3.7***	0.65	3.3***	0.57	1.1
Tis × Gen × Har	30	0.38	2.5***	0.40	2.1**	0.60	3.0***	1.04	1.9**
Tis × Tre × Gen × Har	30	0.30	2.0**	0.28	1.5*	0.40	2.1**	0.85	1.6*
Error within	432	0.15		0.19		0.20		0.53	

[a] $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

infection and, counterintuitively, in a reduced C/N ratio. The lower C/N ratio was associated with reduced levels of the carbon-based secondary metabolites aucubin and catalpol and increased feeding by leaf-chewing insects. Although these results are open to alternative interpretations, they illustrate the possibility that infection by below-ground organisms can result in a systemic increase in the levels of iridoid glycosides in above-ground plant tissues. Induction of iridoid glycosides, thus, seems to be a generalized response to challenge by different biotic agents.

In the present study, we were unable to show that higher constitutive and/or induced levels of iridoid glycosides were associated with enhanced resistance to the fungal pathogen *D. adunca*. This is at variance with recent results from a more extensive set of inoculations of 70 full-sib families that had been artificially selected for high and low constitutive levels of iridoid glycosides (Marak, 2000), showing that growth and reproduction of this fungus are reduced on plants selected for high

TABLE 4. NUMBER OF SEEDS PER MILLIMETER OF SPIKE (± 1 SE) PRODUCED BY THREE *P. lanceolata* GENOTYPES, P1, P2, AND P3, AFTER INOCULATION WITH *D. adunca* (INOC.) AND ON UNINOCULATED CONTROLS (CONTR.)[a]

	P1		P2		P3	
Plant genotype	*N*	Mean (s.e.)	*N*	Mean (s.e.)	*N*	Mean (s.e.)
Seeds (*N*) mm (contr.)	5	1.69 (0.18) a	5	2.27 (0.17) b	5	2.51 (0.18) b
Seeds (*N*) mm (inoc.)	5	0.00 (0.00) a	5	0.00 (0.00) a	5	2.21 (0.17) b
Infection (%)	23	100.0 a	24	100.0 a	23	73.9 b
Latent period (days)	23	10.36 (0.54) a	24	11.28 (0.45) a	17	19.10 (1.36) b
Growth rate (mm/day)	23	9.21 (0.56) a	24	10.38 (0.35) a	17	10.03 (0.39) a
Pycnidia density	23	1.47 (0.08) a	24	1.18 (0.09) b	17	0.35 (0.08) c

[a] The percentage of inoculated plants that became infected, and back-transformed mean values of latent period, growth rate, and an index for the density of asexual fruiting bodies (pycnidia) of the fungus on infected plants are indicated as well. *N*: sample size. Means within a row that do not share a common letter are significantly from each other ($P < 0.05$).

constitutive levels of iridoid glycosides compared to plants selected for low levels of iridoid glycosides. Such an association was predicted on the basis of the *in vitro* antimicrobial activity of the aglycones of aucubin and catalpol (Rombouts and Links, 1956; Ishiguro et al., 1982; Van der Sluis et al., 1983; Davini et al., 1986; Stermitz, 1988, Marak et al., 2002), assuming that the pathogen indeed encounters the iridoid glycosides in their hydrolyzed, aglycone, form. Due to the inherent difficulty of quantitatively detecting these instable iridoid aglycones (Davini et al. 1986; Stermitz, 1988), it is still an open question whether the latter actually occurs and whether, in our experiment, pathogen infection indeed resulted in increased levels of the iridoid aglycones that are required for antimicrobial activity and hence for induced resistance. The fact that, in contrast to the study by Marak (2000), no association between iridoid glycoside levels and resistance was observed in the present study suggests that other factors are involved in resistance as well and that a larger sample of genotypes than the three used in the present study is required to detect such associations. The detection of the negative association between iridoid glycoside levels and resistance in the larger set of genotypes implies that induction can potentially result in increased resistance to the fungus, provided that resistance is still a monotonically increasing function of iridoid glycoside level at high concentrations of these compounds and that the fungus encounters the iridoids at least partly as their aglycones. However, such benefits have yet to be shown. Preliminary studies by C. Jansen (NIOO, Heteren) indicate that when the first flowering stalk on a plant is inoculated, fungal growth on the succeeding stalk, inoculated 1 week later, is slower (21.4 ± 5.7 cm in 5 weeks) than on the first stalk (28.7 ± 5.8 cm; pairwise t test, $t[30] = 6.4$, $P < 0.001$). However, this appears to be an ontogenetic effect rather than an effect of induced resistance, since fungal

growth rates on succeeding stalks of control plants, on which the first stalk was not inoculated, were similarly low (19.9 ± 5.0 cm) as growth rates on the succeeding stalks of plants where the first stalk was inoculated ($t[24] = 1.0$, $P = 0.31$).

Given the broad inducibility of aucubin and catalpol and their potential effects on both arthropods and microorganisms, there is also a potential for reciprocally induced cross-resistance between fungal pathogens and generalist insect herbivores through altered levels of iridoid glycosides in *P. lanceolata*. As discussed by Krischick (1991), there are several implications of such inducible "generalized" defense compounds. First, attack by one type of phytophage could partly determine the suite of phytophages that can subsequently colonize the host and hence indirectly affect the population dynamics of these organisms, as well as local community structure. Second, the resulting plant-mediated interactions between phytophages may affect the type of coevolutionary relationships between plants and phytophages. Since successful attack by one type of phytophage affects the resistance of the host plant to an ensuing, unrelated phytophage, plants susceptible to the first phytophage will have a higher probability of being induced and show increased resistance to the succeeding phytophage. The outcome of selection on resistance to these phytophages is, therefore, no longer independent of each other and hence neither of the phytophages is likely to develop a pairwise coevolutionary relationship with its host but, instead, coevolution is likely to be diffuse (Hougen-Eitzman and Rausher, 1994).

Genotype- and Tissue-Specific Induction of Iridoid Glycosides. Induced defense can only evolve by natural selection if there is phenotypic variation in the level of induction that affects plant fitness and if at least part of that variation has a genetic basis. Heritable variation for the level of induction has previously been reported for 14 natural plant–herbivore and one natural plant–pathogen system (Agrawal, 2000), indicating that, in general, there is potential for a response to selection on levels of induced defense. We found highly significant genotypic differences in the level of induction of iridoid glycosides following challenge of *P. lanceolata* with the pathogenic fungus *D. adunca*. This is in accordance with earlier studies reporting genotypic variation in induction levels in this species following challenge by herbivores (Bowers and Stamp, 1993). The two genotypes with relatively low levels of iridioid glycosides in their spikes both showed induction in this tissue type, whereas the genotype with the highest constitutive level of iridoid glycosides in its spikes showed no induction at all. However, obviously, the sample size of three genotypes is insufficient to address the question of whether there is a general pattern indicating a genetically based trade-off between constitutive and induced defense, as postulated in some evolutionary models of plant defense (Karban and Baldwin, 1997). Our study further shows that in addition to genotypic variation in the average levels of induction, there is also heritable variation for the tissue-specific allocation pattern of induction of iridoid glycosides, as evidenced by the interactions between tissue, genotype, and induction treatment.

This offers scope for further evolutionary fine-tuning of induced responses at the level of specific plant tissues in this system. For instance, there could be an evolutionary response to selection favoring high levels of induction in vegetative parts as opposed to reproductive parts or *vice versa*.

In agreement with a previous study in *P. lanceolata* (Darrow and Bowers, 1999), we found that constitutive levels of iridoid glycosides were higher in reproductive tissues than in vegetative plant parts. Since we lack knowledge of the incidence of attack of different plant tissues in the field and the fitness loss incurred by damage of different tissue types, it is difficult to judge whether this allocation pattern should be interpreted as adaptive or not (Rhoades, 1979; Zangerl and Berenbaum, 1990). Interestingly, the average levels of induction of aucubin and catalpol following challenge by the stalk pathogen *D. adunca* showed a tissue-specific pattern that was similar to the pattern observed for constitutively produced iridoid glycosides: both the absolute and the relative increase in the levels of these compounds in challenged plants was higher in reproductive tissues than in vegetative plant parts. Induction by the stalk pathogen strengthened the existing allocation pattern of constitutively produced iridoid glycosides present in uninduced plants. The similarity of these patterns may reflect a common principle, such as tissue-specific production, decay, or translocation of iridoid glycosides. Alternatively, the observed high level of induction in reproductive organs may be due to factors that are completely unrelated to those governing high constitutive levels of iridoid glycosides in these tissues. Inoculations took place in the spikes, and the level of induction often shows a diminishing response away from the site of attack (Tuomi et al., 1988), which would result in the observed decrease in average induction levels from spikes to stalks, leaves, and roots. The latter explanation is supported by the fact that there is no indication of higher levels of induction in reproductive tissues if herbivory is mainly concentrated in leaves (Darrow and Bowers, 1999). Plants of *P. lanceolata* that were grown at low levels of fertilizer and that were subjected to a 3-day period of herbivory by the specialist leaf chewing insect *J. coenia*, showed ca. 65% induction in the leaves, the site of attack, and ca. 55% in stalks and roots, 4 weeks after termination of the herbivory treatment. One day after herbivory, none of the induction levels were yet significant, the average levels of induction being 25% in leaves, 15% in stalks, and none in the roots (Darrow and Bowers, 1999). The high levels of induction in reproductive tissues observed in the present study may thus reflect the location of attack rather than a general allocation pattern of induction levels.

In general, reproductive plant parts appear to be less inducible than vegetative tissues (Baldwin and Karb, 1995). Karban and Baldwin (1997) speculated that the paucity of studies reporting induction of defense chemicals in reproductive tissues might be due not only to the much larger number of studies that have focused on induction in leaves compared to reproductive parts, but also to the fact that high constitutive levels of defense chemicals might be a more effective way to protect

such valuable tissues that are difficult to replace, than the time-lagged mechanism of induction. An inducible system of defenses for these tissues would provide little additional benefit to plants with constitutively defended reproductive parts. Indeed, in wild parsnip (*Pastinaca sativa),* a monocarpic species in which loss of flowering parts incurs a direct fitness cost (Nitao and Zangerl, 1987) and reproductive parts are the most heavily damaged tissues in the field, fruits show the highest constitutive levels of furanocoumarins but the lowest levels of induction of these defense chemicals (Zangerl and Berenbaum, 1990; Zangerl and Rutledge, 1996). By contrast, our study indicates that constitutively defended reproductive tissues do not necessarily show low levels of induction. We speculate that in *P. lanceolata*, by virtue of the numerous flowering stalks that are produced sequentially during the season, time-lagged systemic induction in reproductive tissues might still protect a significant part of the annual reproductive output of the plant, provided that there is a monotonic increase in the contribution of iridoid glycoside levels to resistance beyond the constitutive levels present in these tissues.

In conclusion, *P. lanceolata* shows heritable variation for the levels of induction of iridoid glycosides, as well as for the within-plant distribution pattern of induction levels following pathogen attack. Since iridoid glycosides can not only deter arthropods and microorganisms, but also be systemically induced by these organisms, they can potentially mediate pathogen-induced resistance to herbivores and *vice versa*. Studies are underway to test whether such effects can actually be observed, or whether they are mitigated by effects of other induced changes in the plant that are bound to occur in response to attack (Paul et al., 1999).

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COMPARATIVE PHYSIOLOGICAL RESPONSES IN CHINESE CABBAGE INDUCED BY HERBIVORY AND FUNGAL INFECTION

MICHAEL ROSTÁS,[1] RICHARD BENNETT,[2] and MONIKA HILKER[1,*]

[1]*Freie Universität Berlin, Institut für Biologie*
Angewandte Zoologie/Ökologie der Tiere
Haderslebener Str. 9, D-12163 Berlin, Germany

[2]*Institute of Food Research*
Norwich Research Park
Colney, Norwich NR4 7UA, United Kingdom

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Abstract—Fungal infection of Chinese cabbage leaves by *Alternaria brassicae* has earlier been shown to have detrimental effects on larval development of the chrysomelid beetle *Phaedon cochleariae*. Furthermore, adults of this leaf beetle avoid fungus-infected Chinese cabbage leaves for oviposition and feeding. However, herbivory had no impact on fungal growth. In this study, we investigated physiological responses of the host plant to herbivore attack and fungal infection in order to elucidate the mechanisms of the described ecological interactions between the fungus and the herbivore. Changes in primary factors (water, C/N ratio, protein, sucrose) and defense-related plant compounds (glucosinolates, anthocyanins, peroxidase) were measured. Herbivory and fungal infection reduced the sucrose concentration of leaves and increased amounts of indole glucosinolates as well as total anthocyanins. In addition, water content was slightly lower in insect-damaged but not in infected leaves. Higher levels of peroxidase activity resulted exclusively from fungal infection. The C/N ratio and total protein content remained unaffected by either treatment. The implications of the induced plant changes on the herbivore are discussed.

Key Words—Tripartite interactions, phytopathogenic fungus infection, herbivory, induced resistance, cross-effects, nutrients, glucosinolates, anthocyanins, peroxidase, Chinese cabbage, *Phaedon cochleariae*, *Alternaria brassicae*.

* To whom correspondance should be addressed. E-mail: hilker@zedat.fu-berlin.de

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INTRODUCTION

Plants have to cope with a multitude of attackers such as pathogenic fungi and herbivores. Damage to plant tissue caused by these antagonists may have a profound impact on host physiology (Hatcher and Ayres, 1997). The alterations of the plant's physiology may be the direct result of fungal growth (involving, e.g., the release of phytotoxins and phytohormones by the parasite) or of wounding by the herbivore. Very often, however, pathogens and herbivores also evoke a variety of active plant responses, including the induction of defensive secondary compounds and shifts in primary metabolism, so-called "civilian responses," which may enable the plant to fend off or to tolerate attackers (Baldwin and Preston, 1999; Hammerschmidt, 1999). Certain plant responses may be induced specifically by either pathogens or herbivores, while other responses lack specificity with regard to the inducing agent. Likewise, the effects of plant responses on the challenge organisms also can be specific (Karban and Kuc, 1999; Stout and Bostock, 1999). However, little is known about the integration of plant reactions to multiple enemies in spite of a wealth of studies concerning inducible responses to either pathogens or herbivorous arthropods (Paul et al., 2000). In the field, herbivorous insects and plant pathogenic fungi frequently co-occur on the same host plant. Therefore, fungi and herbivores may interact indirectly, with the interaction mediated by the induced physiological changes of the host plant. The relationship between the antagonists may be beneficial or detrimental to one or both sides (Hatcher, 1995).

The ecological effects of such tripartite interactions have previously been studied in a system including the crop plant *Brassica rapa* ssp. *pekinensis* (Chinese cabbage), the leaf beetle *Phaedon cochleariae*, and the necrotrophic fungus *Alternaria brassicae* (Rostás and Hilker, 2002a,b). Fungal growth was not affected by feeding damage caused by larvae of *P. cochleariae*, but larval performance was impaired when larvae were fed with parts of Chinese cabbage leaves without symptoms of fungus infection. Larvae feeding on infected Chinese cabbage leaves suffered reduced growth rates. Furthermore, the adults were shown to respond to fungal disease of their host plant. Female beetles avoided diseased leaves for feeding and oviposition.

The present study investigated several traits of plant primary and secondary metabolism of herbivore-damaged and fungus-infected leaves of Chinese cabbage that might be relevant for these observed ecological effects of fungus-infected leaves on larval development and adult behavior in *P. cochleariae.* Only few studies have focused on a direct comparison of responses caused by both pathogens and herbivores on the same host plant (e.g., Stout et al., 1999).

In particular, we assessed: leaf water content, C/N ratio, total proteins, sucrose concentration, concentrations of individual glucosinolates, total anthocyanin concentrations, and peroxidase activity in herbivore-damaged and fungus-infected Chinese cabbage leaves.

Among the various factors that can change the suitability of a host plant for insect consumers is the water content of leaves (Slansky and Scriber, 1985). Pathogenic fungi often enhance the dry matter-to-water ratio of the infected plant by an increase of fungal tissue, by inducing plant cell wall enforcements, or by destroying the leaf epidermis (Duniway and Durban, 1971; Hatcher, 1995; Götz, 1996).

The carbon-to-nitrogen (C/N) ratio is a general indicator of food quality as it may inform about the availability of nutritionally valuable nitrogen in relation to indigestible structural carbohydrates. Growth efficiency of many insect species is correlated with plant nitrogen content (Schoonhoven et al., 1998). A high C/N ratio can, therefore, be detrimental to herbivores (Clancy, 1992). Shifts in carbon and nitrogen concentrations may be caused by the whole spectrum of primary and secondary plant responses following fungal infection and herbivory. These changes may be due to increased photosynthesis, reallocation of storage compounds, the synthesis of proteins or nitrogen-containing allelochemicals, as well as many other responses of the plant (Ayres, 1992; Baldwin and Preston, 1999).

Additionally, we analyzed total protein content as the most important nutrient for herbivorous insects. The concentration of sucrose in herbivore-damaged and fungus-infected leaves was also measured, because sucrose is known as a phagostimulant for several leaf beetles and other insects (Dadd, 1985; Matsuda, 1988). In addition to sucrose, glucosinolates may stimulate feeding in crucifer specialists like the leaf beetle *P. cochleariae* (Nielsen, 1978; Wallsgrove et al., 1999; Fahey et al., 2001). However, the stimulatory effect of glucosinolates may be dose-dependent: specialists have been shown to feed less on individuals of *Brassica rapa* that contained higher glucosinolate levels than on plants of the same population with low glucosinolate concentrations (Siemens and Mitchell Olds, 1996). Glucosinolates are known to be inducible by plant pathogenic fungi and herbivorous insects (Ludwig-Müller et al., 1997; Hopkins et al., 1998). They may also be induced in Chinese cabbage by treating the plants with salicylic acid or jasmonic acid — two compounds that are often involved as wound signals in the defense against pathogens and herbivores, respectively (Ludwig-Müller et al., 1997).

Anthocyanins belong to the large group of flavonoids and are ubiquitous in petals, stems, and leaves of angiosperms. Primarily they are known as the compounds that give flowers their red or blue color or protect the plant against ultraviolet radiation (Harborne and Williams, 2000). However, cyanidin-3-β-glucoside (chrysanthemin) has also been reported as a major factor of resistance in cotton towards *Heliothis virescens* (Hedin et al., 1983). The defensive role of proanthocyanidins (precursors of anthocyanins) against herbivores has been established (Harborne and Williams, 1995).

Finally, we measured the activity of soluble leaf peroxidases (POX). These are widespread plant enzymes that use hydrogen peroxide to oxidize phenolics and other substrates. They are considered to contribute to plant defense against herbivores and fungi in several ways: products from phenolic oxidation may be

cytotoxic, bind to dietary proteins, and inactivate fungal proteins. Additionally, they can contribute to cell wall reinforcement that results in greater leaf toughness (Dowd and Lagrimini, 1997). Increased activity of peroxidases may be induced by herbivore damage and by pathogenic infection. However, the biological functions of peroxidases *in planta* have been difficult to verify and are not yet resolved (Constabel, 1999; Østergaard et al., 2000).

In this study, the above-mentioned physiological parameters are compared between fungus-infected and healthy plants, as well as between herbivore-damaged and unattacked plants. Differences are discussed with respect to the described ecological effects of the tripartite interactions among Chinese cabbage, the fungus *A. brassicae*, and the leaf beetle *P. cochleariae.*

METHODS AND MATERIALS

General. Chinese cabbage plants (*Brassica rapa* L. ssp. *pekinensis* cv. Kantonner) were grown from seed (obtained from Saatzucht Quedlinburg GmbH) in 7- × 8- × 7-cm plastic pots in a greenhouse at ca. 20°C. Standard potting soil (Einheitserde Typ P, Kausek GmbH and Co. KG, Mittenwalde, Germany) was used and one additional dose of fertilizer was applied three weeks after germination. Light from 400-W sodium vapor lamps supplemented natural daylight, providing an illumination of >10,000 lx for 16 hr a day. Plants were assigned to herbivore or fungus treatment when the fifth (first leaf = oldest leaf) had reached maximum size (4–5 weeks). Only the fifth leaf was used for analyses.

Strain BBA 64878 of *Alternaria brassicae* (Berk.) Sacc., the causal organism of blackspot disease, was obtained from the Biologische Bundesanstalt (Berlin-Dahlem, Germany). The fungus was cultured on potato–dextrose agar (PDA) at 20°C without induction of sporulation. Cultures used for inoculation were 5 days old.

A laboratory colony of *Phaedon cochleariae* (F.) was maintained in a climate chamber at 20°C, 70% relative humidity, and a photocycle of 18L:6D. Larvae and adults were fed on Chinese cabbage leaves.

Herbivore Treatment and Fungal Inoculation of Plants. Experimental plants were selected for equal size and arranged in a completely randomized design. A clip cage (21 mm diam., 18 mm high) containing five second-instar beetle larvae was attached to the fifth leaf of an experimental plant. Larvae were allowed to feed for 72 hr in total, removing about 9% of the leaf area. After periods of 24 and 48 hr, the clip cage with the larvae was shifted to an undamaged part of the leaf. Following this treatment, the leaf was excised and used for analyses (see below). Control plants received identical treatment with an empty clip cage.

For the inoculation of Chinese cabbage with the fungus *A. brassicae*, a method described by Gröntoft and O'Connor (1990) was adopted. The fifth leaves of Chinese cabbage plants were inoculated with small mycelium-containing agar

disks (5 mm diam.) punched out from behind the growth front of Petri dish cultures using a cork borer. Two disks were laid with their mycelium-containing side onto the adaxial surface of a leaf half. Upon each disk, and underneath it on the abaxial side of the leaf, a piece of firm foam material ($10 \times 10 \times 7$ mm) was placed. An insect needle (No. 00) was pierced through inoculum, leaf, and foam material, fixing the "sandwich" construction. Control plants were treated equally, but agar disks contained no mycelium. Following inoculation, treated plants and controls were placed under a tent made of thin transparent plastic foil in a completely randomized design for 5 days. The tent ensured an atmosphere of 100% relative humidity, thus enhancing fungal growth. Harvested leaves were cut along their midveins, and the leaf halves bearing no symptoms of fungal infection were used for chemical analyses.

Chemical Analyses. The fifth leaves of herbivore-damaged and fungus-infected plants and their corresponding controls were detached and immediately submerged in liquid nitrogen. Samples were then stored at -70°C until needed. Freeze-dried leaf tissue powder was used in all analyses except for measurements of water content and POX activity. For further preparation, samples were lyophilized for 3 days and ground in a planetary micromill. All spectrophotometric analyses were carried out using an UV–visible spectrophotometer (Ultrospec, 2000, Pharmacia Biotech, Freiburg, Germany). Samples from five plants were assessed in triplicate if not otherwise stated.

Analyses of Primary Compounds. The water content of herbivore-damaged and pathogen-infected leaves was determined from the fifth leaves of 20 plants from each treatment group. Leaves were excised, and the succulent petioles and midveins were removed. The remaining laminas were weighed and then left to dry at 80°C for 2 days. Dried leaves were weighed again and percentage water content was calculated.

Total carbon and nitrogen content was analyzed using a CHN analyzer (Leco CHN-932, Typ 600-800-532). Two milligrams of tissue powder were weighed into 50-mg tin capsules. Carbon and nitrogen concentrations were determined in duplicate.

Total protein content was determined after Bradford (1976) and Jones et al. (1989). Thirty milligrams of lyophilized tissue powder was added to 2 ml 0.1 N NaOH. The suspension was agitated on a vortex mixer for 3 sec, left to extract for 30 min, agitated again for 3 sec, and then centrifuged for 5 min at 12,000g. The supernatant was decanted and vortex mixed for 3 sec. Aliquots of 100 μl of the supernatant or 100 μl of 0.1 N NaOH (blank) were each mixed with 1 ml Bradford dye reagent to which 3 mg/ml soluble polyvinylpyrrolidone (PVP; MW: 40,000) had been added. The absorbance of the leaf extract was read at 595 nm against the dye reagent/NaOH blank. Bovine serum albumin was used as a standard.

Sucrose concentrations of treated and control Chinese cabbage leaves were determined enzymatically. A commercial test kit was used (Boehringer Mannheim

GmbH, Biochemica, Mannheim, Germany; no. 716 260). Thirty milligrams of lyophilized tissue powder were extracted for 1 hr in 2 ml aq. bidest. The homogenate was then centrifuged for 15 min at 12,000*g*, and the supernatant was transferred into 1.5-ml vials. Enzymatic hydrolysis of sucrose was done according to the test kit manual except that only a quarter of the suggested quantities of all buffers and enzyme suspensions was used. Extinction was read at 340 nm.

Analysis of Secondary Compounds. Glucosinolates were extracted and measured as described by Kiddle et al. (2001). Essentially, samples (3×40 mg for each sample) of tissue powder were extracted with 70% v/v MeOH. The extract was bound to preactivated Sephadex A-25, and the glucosinolates were desulfated with purified *Helix* sulfatase for at least 16 hr. The desulfoglucosinolates were eluted with water and stored at -20°C until analyzed by reverse-phase HPLC (Kiddle et al., 2001). Sinigrin was used as the internal standard.

Concentrations of total anthocyanins were analyzed colorimetrically (Manicelli et al., 1984). Tissue powder (3×10 mg for each sample) was vortex mixed with 1 ml of 1% v/v HCl in 100% MeOH in a screw-top microtube. Samples were centrifuged at 4°C for 10 min at 17,000*g*. The supernatant was then carefully removed and absorbance of anthocyanins was measured at 530 nm. A second reading at 657 nm assessed contaminating chlorophylls that absorb in the 530 nm region. Anthocyanin specific absorption unit was $AU = A_{530} - A_{657}$.

To assay peroxidase (POX) activity, 1 g of fresh foliage was homogenized in 2 ml of 50 mM ice-cold sodium phosphate buffer (pH 7.0, containing 0.5 mM EDTA, 5 mM sodium-metabisulfite, and 40 ml/liter protease inhibitor cocktail, Sigma, Deisenhofen, Germany; P 2714). Acid-washed sand (100–200 mg/g tissue) in the mortar improved homogenization. The homogenate was added to 1.25% w/v polyvinylpolypyrrolidone (PVPP) and 1.25% w/v amberlite XAD-4 in a beaker (25 ml) and left on ice for 5 min. The homogenate was then filtered through a muslin cloth into a prechilled centrifuge tube and centrifuged at 4°C for 20 min at 14,000*g*. The supernatant was removed and immediately used as the enzyme source. A 50-μl aliquot of foliar homogenate was assayed with 1 ml of 10 mM 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) and 10 mM H_2O_2 (30% v/v) in 50 mM NaH_2PO_4 (pH 6.0). POX activity was estimated at 414 nm and 25°C.

Statistics. Student's *t* tests were used to analyze differences between herbivore-damaged or fungus-infected leaves and their corresponding controls. Due to differing growth conditions (see above) statistical testing was not performed between herbivore-damaged and fungus-infected leaves.

RESULTS

Analyses of Primary Compounds (Table 1). Water content of Chinese cabbage leaves significantly decreased after 3 days of herbivory by second instars

TABLE 1. PRIMARY COMPOUNDS MEASURED IN HERBIVORE-DAMAGED AND FUNGUS INFECTED CHINESE CABBAGE LEAVES AND THEIR RESPECTIVE CONTROLS[a]

	Treatment			
	Herbivory	Control	Fungus	Control
Water content [%]	89.8 (1.59)***	91.4 (0.68)	91.6 (0.81)	91.7 (0.63)
C/N	6.6 (0.54)	7.8 (1.29)	6.1 (0.82)	6.0 (0.24)
Total protein (mg/g foliar dry weight)	61 (4.0)	59 (2.3)	57 (6.7)	57 (7.7)
Sucrose (g/100 g foliar dry weight)	1.5 (0.34)*	2.2 (0.61)	1.2 (0.52)*	2.2 (0.92)

[a] Herbivory = leaves damaged for 72 hr by second instars of *Phaedon cochleariae*. Fungus = leaves 5 days after infection with *Alternaria brassicae*. $N = 5$ (water content: $N = 20$). Values are means (standard deviations). Asterisks denote significant differences (*** $P < 0.001$, * $P < 0.05$; Student's t test for independent samples).

of *P. cochleariae*. However, the reduction did not exceed 2%. In contrast, leaves infected by *A. brassicae* did not differ ($P = 0.847$) in their mean water content from healthy control leaves. The C/N ratio did not significantly differ in leaves receiving either herbivore ($P = 0.096$) or fungus ($P = 0.835$) treatment when compared with their respective controls. Total protein content of Chinese cabbage leaves did not significantly change as a consequence of herbivore or fungal activity (herbivore: $P = 0.376$; fungus: $P = 0.773$). Herbivory and plant pathogenic infection reduced sucrose content as indicated by a 31% loss of foliar sucrose in the case of herbivore-damaged leaves and a 47% decrease in the pathogen-infected leaves (herbivore: $P = 0.018$; fungus: $P = 0.035$).

Analyses of Secondary Compounds. Production of glucosinolates was induced in herbivore-damaged and in fungus-infected leaves (Figure 1). Chinese cabbage leaves on which larvae of *P. cochleariae* had fed showed a 2.6-fold increase in total glucosinolate content compared with untreated control leaves ($P < 0.001$). Glucosinolate content of leaves infected with the fungus *A. brassicae* was 5.9 times higher than healthy tissue ($P < 0.001$). This effect was entirely due to an increase in the concentrations of indole glucosinolates (Figure 1). Glucobrassicin (indol-3-ylmethyl glucosinolate) was 12.3 times higher in herbivore-damaged leaves, while the increase in fungus-infected leaves was 11.6-fold (herbivore: $P = 0.004$; fungus: $P = 0.008$). An accumulation of 4-MeO-glucobrassicin (4-methoxy indol-3-ylmethyl glucosinolate) was observed in leaves treated with the fungus (3.5-fold, $P \leq 0.001$). In herbivore-damaged leaves, a slight increase of about 10% was measured; however, this was not significantly different from the control ($P = 0.693$). Elevated concentrations of neoglucobrassicin (1-methoxy indol-3-yl-methyl glucosinolate) were found in both treatment groups (herbivore: 2.2-fold, $P = 0.027$; fungus: 5.9-fold, $P = 0.002$). Aliphatic glucosinolates were measured only in trace amounts, and these were not affected by either treatment. Concentrations of aromatic glucosinolates were below detectability.

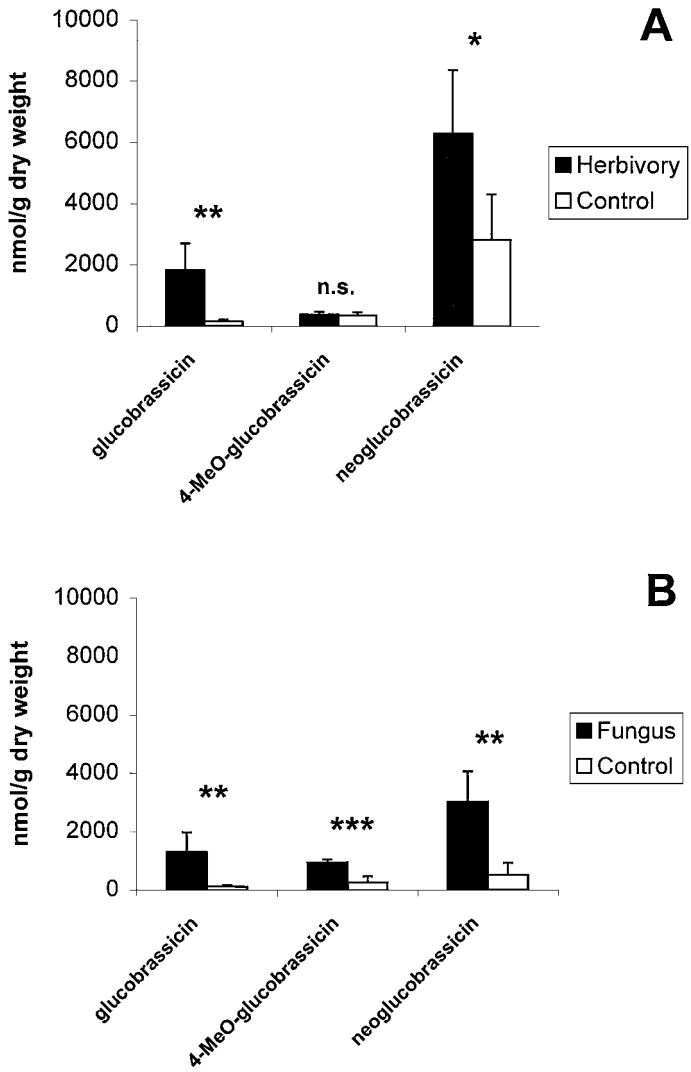


FIG. 1. Concentrations of indole glucosinolates in herbivore-damaged (A) and fungus infected (B) Chinese cabbage leaves. Herbivory = leaves damaged for 72 hr by second instars of *Phaedon cochleariae*. Fungus = leaves 5 days after infection with *Alternaria brassicae*. $N = 5$. Values are means and standard deviations (bars). Asterisks denote significant differences ($^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, n.s. = not significant; Student's t-test for independent samples).

TABLE 2. SECONDARY COMPOUNDS MEASURED IN HERBIVORE-DAMAGED AND FUNGUS INFECTED CHINESE CABBAGE LEAVES AND THEIR RESPECTIVE CONTROLS[a]

	Treatment			
	Herbivory	Control	Fungus	Control
Rel. anthocyanin conc. (AU $\times 10^3$)	271 (31.4)***	36 (6.1)	173 (35.3)***	34 (4.9)
POX ($dA_{414} \times 10^2$/min/g foliage)	326 (106.6)	356 (106.2)	1482 (418.0)***	544 (148.5)

[a] Herbivory = leaves damaged for 72 hr by second instars of *Phaedon cochleariae*. Fungus = leaves 5 days after infection with *Alternaria brassicae*. $N = 5$. Values are means (standard deviations). Asterisks denote significant differences (*** $P < 0.001$; Student's t test for independent samples).

Total anthocyanin content was 7.5 times higher in leaves affected by herbivory when compared with undamaged controls ($P < 0.001$). An increase in the anthocyanin concentration by a factor of 5.1 was observed in fungus-infected leaves ($P < 0.001$) (Table 2).

Activity of POX assayed with ABTS as the hydrogen donor differed in herbivore and fungus treated plants (Table 2). Previous feeding by the herbivore did not result in increased POX activity ($P = 0.767$) in Chinese cabbage leaves. However, POX activity was enhanced in leaves infected by the fungus compared to the control treatment (2.7-fold, $P < 0.001$).

DISCUSSION

In this study, we showed that herbivory and fungal infection to some extent induced similar but also differential responses in leaves of Chinese cabbage.

The water content of leaves on which larvae of *P. cochleariae* had fed for 3 days was significantly reduced. It seems obvious that wounding of leaf tissue leads to an increased loss of water. However, this reduction (−1.6%) might be negligible for the beetle larvae, as the total water content of healthy Chinese cabbage leaves was high (>90%, Table 1). Consequently, no signs of wilting were observed. Fungus-infected leaves, on the other hand, did not differ in their water content from healthy leaves. This is consistent with studies that measured the leaf water content of fungus-infected plants in conjunction with herbivore consumption (Kingsley et al., 1983; Hatcher et al., 1994, 1995).

No shifts in the C/N ratio of Chinese cabbage leaves were observed following herbivory or fungal infection. The carbon and nitrogen contents were comparable to those obtained from analyses of commercially grown Chinese cabbage plants (Müller, 1999). Likewise, neither treatment altered the total protein content in the leaves. In contrast, sucrose concentrations were lower in both wounded and infected leaves. It is conceivable that in the case of herbivore-damaged leaves the decrease in sucrose concentration might be a genuine plant response. Exporting

sucrose may be a mechanism for reallocation of valuable nutrients to other unaffected plant parts for compensatory growth. This has been hypothesized for the observed reduction in the concentrations of total free sugars in herbivore-damaged oak leaves (Valentine et al., 1983). The lower sucrose content of the uninfected half of a fungus-infected leaf is most likely due to an altered sink-source relationship caused by the pathogen. Köhle (1989) showed that radiolabeled [^{14}C] sucrose accumulated only in those halves of oilseed rape (*Brassica napus*) leaves that were infected with *Alternaria brassicicola.* Changes in leaf nutrients caused by the activity of herbivores and fungi may vary greatly depending on several factors such as the timing and intensity of feeding damage or infection and the system that has been studied (Hatcher, 1995; Karban and Baldwin, 1997). An increase in carbohydrates other than sucrose, in wounded and infected leaves, may have contributed to the stable C/N ratio after pathogen or herbivore attack.

Following herbivory and fungal infection, a distinct rise in the concentrations of the indole-glucosinolates glucobrassicin, 4-MeO-glucobrassicin, and neoglucobrassicin was observed. Constitutive levels of glucosinolates were higher in control plants of the "herbivore" treatment group than in the "fungus" treatment group. This was probably due to different treatment conditions. Plants inoculated with *A. brassicae* were kept in a tent with higher humidity and higher temperatures. However, a more pronounced induction of glucosinolates was observed in the infected plants. Differing responses were found with regards to 4-MeO-glucosinolate: this compound was induced in leaves infected with the fungus, but not in plants damaged by herbivory. Koritsas et al. (1991) demonstrated similar effects in the stem tissue of *B. napus* cv. Ariana that had been damaged by the chrysomelid beetle *Psylliodes chrysocephala.* Aerial infestation stimulated the production of glucobrassicin (6-fold), 4-MeO-glucobrassicin (2-fold), and neoglucobrassicin (23-fold), while levels of aliphatic and aromatic glucosinolates remained too low to be detected. Accumulation of glucosinolates was also observed in leaves of *B. napus* (cv. Cobra, cv. Bienvenue) following *A. brassicae*-infection (Doughty et al., 1991). The increase was mainly the result of higher levels of indole and aromatic glucosinolates, while concentrations of aliphatic glucosinolates were variable.

Chinese cabbage leaves responded to feeding damage and fungal infection, respectively, by raising the levels of total anthocyanins. Accumulation of anthocyanins is induced by a range of stimuli including UV light, low temperature, phytohormones, and pathogens (Lo and Nicholson, 1998). Interestingly, six-carbon (C_6) volatiles and methyl jasmonate, which are released by insect-damaged plant tissues, have also been demonstrated to induce the production of anthocyanins (Franceschi and Grimes, 1991; Bates and Rothstein, 1998). These volatiles play a role in the plant's defense response against herbivores and may activate the expression of phenylpropanoid-related genes and the production of proteinase inhibitors (Karban and Baldwin, 1997; Bates and Rothstein, 1998).

A significant increase in the activity of POX in the uninfected half of *A. brassicae*-infected leaves was observed. This contrasted with results obtained from Chinese cabbage leaves damaged by *P. cochleariae*, where no significant difference between treated and control leaves was apparent. In one of the few studies investigating the specificity of host plant responses to herbivores and pathogens, the same pattern was evident (Stout et al., 1999). Tomato plants inoculated with the pathogenic fungus *Phytophthora infestans* had increased POX activities. Wounding by *Helicoverpa zea*, on the other hand, showed no such effects. However, the induction of POX by insect feeding is not uncommon and has been demonstrated in tomato by other insects and also in cotton (Bi et al., 1997; Stout et al., 1998). Enhanced POX activity due to infection by *A. brassicae* or *A. brassicicola* was measured in *B. juncea*, with higher activities found in susceptible cultivars (Gupta et al., 1990).

Considering the observed phytochemical responses, what can be inferred from our results that might explain the adverse effects of plant pathogenic fungus infection on the larval performance of *P. cochleariae*? First, there were no changes in the nutrient status of leaves except for a decrease in sucrose concentration as a result of fungal infection. Second, fungal infection invoked higher levels of indole glucosinolates, anthocyanins, and POX activity. When interpreting these data, it is important to be aware that the larval performance of *P. cochleariae* is not affected when larvae consume leaves that were previously fed upon by conspecific larvae (Rostás and Hilker, 2002a). Consequently, one might suggest that all plant responses equally induced by the beetle and the fungus may be excluded as factors responsible for reduced host plant quality, i.e., changes of the concentrations of sucrose, anthocyanins, glucobrassicin, and neoglucobrassicin (Figure 2). In this study, the compounds exclusively induced by *A. brassicae* are 4-MeO-glucobrassicin and POX. However, it seems unlikely that 4-MeO-glucobrassicin can be considered as a resistance factor, since *P. cochleariae* is a crucifer specialist and, therefore, is expected to be able to detoxify glucosinolates (Louda and Mole, 1991). Enhanced POX activity, on the other hand, is more likely to have contributed to an increased resistance of fungus-infected leaves against the herbivore. In cotton, POX, among other oxidative components, was discussed as a contributing factor in resistance against herbivores (Bi et al., 1997). Likewise, overexpression of POX in transgenic tobacco leaves conferred resistance against *Helicoverpa zea* (Dowd and Lagrimini, 1997).

Which of the measured physiological factors could have affected the behavioral response of adult *P. cochleariae* towards fungus-infected Chinese cabbage leaves and may be relevant for the deterrent effect of diseased leaves? When trying to answer this question, it must be considered that adult *P. cochleariae* are not only deterred by fungus-infected leaves, but also by leaves that have been damaged by conspecific larvae (Rostás and Hilker, 2002b). This implies that although there is some overlap of the plant responses to herbivory and fungal infection (Figure 2),

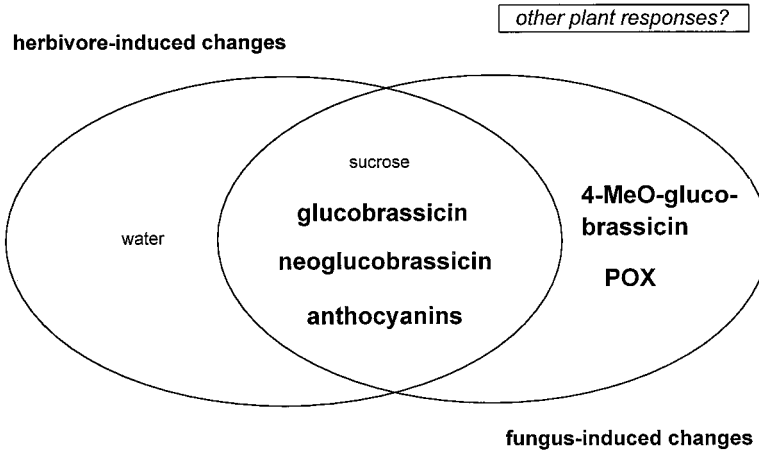


FIG. 2. Overlapping and differential responses of Chinese cabbage leaves due to feeding damage caused by *Phaedon cochleariae* and fungal infection with *Alternaria brassicae*. Compounds written in large bold or small (non-bold) letters showed significant increases or decreases, respectively, in their concentrations due to herbivory or fungal infection compared with corresponding controls.

none of the observed physiological changes can be excluded as putative resistance factors. Two different resistance mechanisms, perhaps with a certain degree of overlap, may have elicited the same reaction in the beetles when confronted with fungus-infected or herbivore-damaged leaves.

Even though the potential role of POX as a factor reducing host plant suitability to *P. cochleariae* is advocated here (see above), it is important to emphasize that plant defenses against herbivores and pathogens are complex phenomena. Certainly, an array of yet unmeasured factors, e.g., fungal toxins, proteinase inhibitors, or phytoalexins, may have also contributed to the resistance effects of the fungus-infected Chinese cabbage plants on the leaf beetle (Bains and Tewari, 1987; Lim et al., 1996; Baur et al., 1998). Future research involving feeding experiments with isolated inducible phytochemicals or transgenic plants will have to elucidate further the underlying mechanisms of plant-mediated cross-effects between *A. brassicae* and *P. cochleariae*.

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PREDATORY RESPONSE OF BROWN TREE SNAKES TO CHEMICAL STIMULI FROM HUMAN SKIN

MICHAEL J. GREENE,* SHANTEL L. STARK, and ROBERT T. MASON

Department of Zoology
Oregon State University
3029 Cordley Hall
Corvallis, Oregon 97331-2914, USA

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Abstract—The brown tree snake (*Boiga irregularis*) is an exotic pest species on Pacific islands, most notably on Guam where it has caused considerable ecological and economic damage. On Guam, the snake commonly associates with people and can be found near or in human habitations. Bites are common, approximately 1 of 1200 emergency room visits to Guam hospitals were reported to be the result of *B. irregularis* bites; 80% of these victims were attacked while sleeping. Most of the attacks occurred on fingers and hands and the attacks appeared to be predatory, rather than defensive, in nature. In order to characterize the mechanism releasing this unusual behavior, we measured the predatory response of *B. irregularis* to chemical stimuli from humans and controls using a lab population that originated from Guam and a wild population from the species' native range in Queensland, Australia. To quantify behavior we measured the proportion of snakes displaying predatory behavior to each of the stimuli, the latency to attack, and the number of tongue-flicks displayed. We quantified predatory behavior using the tongue-flick attack score for repeated measures [TFAS(R)], a common method for quantifying predatory behavior in squamate reptiles. Captive brown tree snakes responded to human skin stimuli with feeding behavior, including predatory attacks, at the same frequency as they did to prey stimuli derived from mice, while never responding to controls with such behavior. Captive snakes also responded to human skin stimuli and prey stimuli with significantly higher TFAS(R) scores than to controls, although there were no differences between the human and mouse stimuli. Wild-caught animals in Australia also responded with predatory attacks to human skin stimuli, while not showing predatory behavior to a blank control and with higher TFAS(R) scores to human skin stimuli than to the control. As *B. irregularis* is a generalized predator that relies heavily on chemical signals to recognize prey,

* To whom correspondence should be addressed at current address: Department of Biological Sciences, Stanford University, 371 Serra Mall, Stanford, California 94305, USA. E-mail: greene@ants.stanford.edu

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we hypothesize that the snakes recognize compounds on human skin that may be shared with other prey.

Key Words—Brown tree snake, *Boiga irregularis*, invasive pest species, predatory behavior, feeding stimulus.

INTRODUCTION

The brown tree snake (*Boiga irregularis*) has caused significant ecological and economic damage on the Pacific island of Guam where it was accidentally introduced during or shortly after World War II (Rodda et al., 1992, 1997). The snake flourished on the island, reaching estimated densities of up to 100 snakes per hectare (Rodda et al., 1992). Pest damage includes power outages caused by snakes climbing onto power lines and short-circuiting the system while searching for prey that result in revenue and equipment losses (Fritts et al., 1987).

A generalist predator, brown tree snakes have caused the extinction or extirpation of 9 of 12 native forest birds from the island of Guam, three of which were endemic (Savidge, 1987). In addition, the snakes have negatively affected domestic animals and pets, rodents, lizards, and the endangered Marianas fruit bat, *Pteropus mariannas* (Wiles, 1987; Savidge, 1988; Greene, 1989; Fritts and McCoid, 1991; Rodda and Fritts, 1992). They feed on carrion in the field (R. T. Mason, personal observation; Shivik and Clark, 1997) and also attempt to ingest their own bodies, cage walls, and items in their cage if prey odors or blood are present (M. J. Greene, personal observation). Incidences of cannibalism have been reported on Guam (Engeman et al., 1996). Noteworthy is the fact that they have been reported to attack and kill animals much larger than they can swallow, such as chickens, newborn pigs, and puppies (Fritts, 1988; Fritts et al., 1994).

The brown tree snake associates with humans, and bites are common; approximately 1 of 1200 visits to Guam hospital emergency rooms were reported to be for brown tree snake bites (Fritts, 1988; Fritts and McCoid, 1999). Eighty percent of bite victims surveyed after being treated at medical facilities were bitten while sleeping (Fritts et al., 1994). Of these victims, 52% were younger than 5 years of age, all of whom were bitten while sleeping (Fritts et al., 1994). Fifty-three percent of the victims 5 years of age and older were bitten while sleeping (Fritts et al., 1994).

Fifty percent of all victims attacked while sleeping were bitten on the fingers and hands while the remaining victims were bitten on the arms, legs, neck, back, and face (Fritts et al., 1994). The bite patterns suggest that the snakes were attempting to ingest the fingers and hands of these victims in the same manner they would a prey item (Fritts et al., 1990). Several cases have been reported where parents have found brown tree snakes chewing on the limbs of their children while coiled around the necks and bodies of the children (Fritts et al., 1990; Fritts and McCoid, 1999).

The brown tree snake is a rear-fanged colubrid containing venom of low toxicity to humans (Weinstein et al., 1991; Vest et al., 1991). The venom is delivered through grooved rear fangs when the snake is making deliberate chewing motions on the body of a prey item (Kardong and Lavin-Murcio, 1993). Envenomation is difficult during defensive strikes because the snakes normally respond to provocation with lunging strikes and quick bites (Johnson, 1975). Defensive strikes are quick and involve the front of the jaw, while predatory bites require slower, more deliberate use of the rear fangs in the posterior of the buccal cavity. Envenomation has been reported when snakes attacked sleeping humans (Fritts et al., 1990, 1994).

No deaths have resulted from brown tree snake bites, although many symptoms requiring medical attention have been reported (Fritts et al., 1990, 1994). The most common were discoloration, swelling, and blebs at the bite location. In addition, high pulse, respiratory arrest, lethargy, and droopy eyelids have been described in several cases (Fritts et al., 1990, 1994). It is rare for snakes to attack humans in a predatory manner; aside from a few large constrictors, kraits (*Bungaris* spp) are the only other snakes reported to attack humans in such a way (Minton and Minton, 1980; Fritts et al., 1990).

Initial observations made with captive brown tree snakes in our laboratory showed that they were attracted to objects touched by human hands. In many instances, tongue-flicking behavior displayed to the human odors on these objects was similar to that displayed when feeding on dead prey items, including short, directed tongue-flicks to the objects. Tongue-flicking in snakes serves to transfer chemicals from the environment to the snake's vomeronasal organ, which detects primarily nonvolatile chemical cues (Halpern, 1992). The experiments presented here were designed to measure the feeding response of *B. irregularis* to human skin chemical stimuli and controls by using a bioassay that is sensitive to, and accounts for, both tongue-flicking behavior and predatory behavior.

METHODS AND MATERIALS

Laboratory Snake Bioassay. Ten adult brown tree snakes (3 females and 7 males) were randomly selected from our captive colony for use. The snakes were originally collected on Guam and housed in our laboratory colony for over 6 years before the experiment (Greene et al., 1997). They were fed a diet of thawed frozen mice and chicks every 2–3 weeks, but were not fed 2 weeks prior to the experiment. Ambient room temperature was 23–30°C (mean: 25°C), and relative humidity was 75–80% in the snake room, simulating conditions on Guam. Lighting (14L:10D) was provided by overhead fluorescent lights and by natural sunlight entering the room through windows.

Four treatments were tested in the experiment: (1) a blank control (untreated cotton swab), (2) a general lipid control (a mixture of 100 m vegetable oil in 5 ml hexane), (3) a mouse treatment (positive control), and (4) a human skin treatment.

The mouse treatment was produced by rubbing a swab on the fur and skin of a thawed house mouse (*Mus musculus*) 10 times. The human skin treatment was created by rubbing a single cotton swab on the skin of the arms of four adult humans (2 females and 2 males) five times each. All swabs were used immediately after preparation. The general lipid control was produced by applying 0.5 ml of the dilute lipid to a swab and allowing the solvent to evaporate.

Experiments were conducted during scotophase when the snakes were normally active following methods patterned after earlier studies by Burghardt (1970, 1975; Burghardt et al., 1988). Snakes were tested in their home cages by placing treated or control cotton swabs in the cage approximately 2-cm from the snake's snout. Treatments were presented in a randomized sequential order in a single session with 15 min between tests. The order of snakes tested was random. In all trials, snakes tongue-flicked the swabs within 2 min, making it unnecessary to stimulate a response by lightly touching the swab to the snouts. Trials in which the snake displayed defensive behaviors to the presence of the swab, such as defensive striking or head-hiding (Johnson, 1975), were suspended and redone during a subsequent session.

Trials began when the focal snake tongue-flicked the swab for the first time. Subsequently, the number of tongue-flicks that made direct contact with the swab was counted for 180 sec or until the snake attempted to ingest the cotton swab (in preliminary observations, the latency to attack dead mice ranged from 7 to 288 sec). The feeding behaviors measured were: (1) close-contact tongue-flicking and (2) attacks. We have only observed close-contact tongue-flicking by *B. irregularis* during feeding events on dead prey items, never during predation on live prey or during defensive attacks (M. J. Greene, unpublished data). The behavior consists of short, directed tongue-flicks at the dead prey item with the snout positioned a few millimeters away in which only the bifurcated tips of the tongue protrude from the mouth.

The tongue-flick attack score for repeated measures [TFAS(R)] was used to quantify the predatory response to the swabs (Cooper and Burghardt, 1990). TFAS(R) is a composite measure (combining tongue-flick frequency and latency to attack data) that weighs trials in which snakes attack the stimulus more heavily than trials in which no attacks occur. $\text{TFAS(R)} = \text{TF} + (\text{TL} - \text{latency})$, where TF is the maximum number of tongue-flicks directed at the swabs for any treatment by the focal snake, TL is the trial length in seconds, and latency is the time in seconds to attack the swab from the start of the trial. Higher scores indicate a greater predatory response (Cooper and Burghardt, 1990).

Wild-Caught Snake Bioassay. This experiment was conducted using brown tree snakes collected from their native range in Queensland, Australia. Immediately after capture, snakes were brought back to a laboratory at the University of Queensland where they were housed and tested. Snakes were housed in captivity for less than 7 days, and all interactions with humans were minimized before

testing. Eight snakes (3 males and 5 females) were tested in their home cages using the same protocol as the laboratory study except that only the human odor and blank control treatments were tested (the mouse and general lipid controls were not conducted with the wild snakes because neither were available in the field at the time of the study). Snakes were tested by using a switchback method; on the first night, snakes were randomly chosen to receive either the control or human odor treatment while receiving the other treatment on the second night.

RESULTS

Laboratory Snake Bioassay. The snakes displayed predatory behaviors more often to the mouse and human skin stimuli swabs than to the control swabs (Cochran's Q test: $Q = 26.2$; $df = 3$; $P < 0.001$; Figure 1A). They responded with predatory behavior in 8 of 10 trials (6 attacks) to human skin stimuli and in 10 of 10 trials (6 attacks) to mouse odor, while never displaying predatory behaviors to either control. TFAS(R) scores were higher for the mouse and human treatments than for the controls (Friedman ANOVA: $Fr = 13.6$; $df = 3$; $P < 0.004$; Figure 2A); however, the response to the human stimulus did not differ from the response to the mouse stimulus ($Fr = 0.001$; $df = 1$; $P < 0.999$). There were no sex differences in TFAS(R) scores for any of the stimuli (Mann-Whitney U test: $P > 0.05$ for all comparisons).

Wild-Caught Snake Bioassay. The results were consistent with laboratory data; the snakes responded to the human skin swabs with predatory behavior in 6 of 7 trials (4 attacks) while never displaying predatory behavior to the control ($Q = 6.00$; $df = 1$; $P < 0.014$; Figure 1B). The snakes had a higher TFAS(R) to human skin stimuli than to the control ($Fr = 7.00$; $df = 1$; $P < 0.008$; Figure 2B). There were no sex differences in TFAS(R) scores for any of the stimuli (Mann-Whitney U test: $P > 0.05$ for all comparisons).

DISCUSSION

Captive brown tree snakes collected on Guam and wild-caught snakes from their native habitat in Australia responded to human skin stimuli with predatory behavior. Specifically, close-contact tongue-flicking and deliberate predatory attacks were displayed to both the human- and mouse-treated swabs (a positive control), while never to the negative controls. These data confirm earlier observations in our laboratory of brown tree snakes investigating items touched by human skin, and they may provide a mechanism to explain why the snakes attack sleeping humans on Guam.

We propose that foraging brown tree snakes accidentally make contact with sleeping humans when searching for natural prey in and around homes on Guam. By tongue-flicking the skin of sleeping humans, the snakes may detect

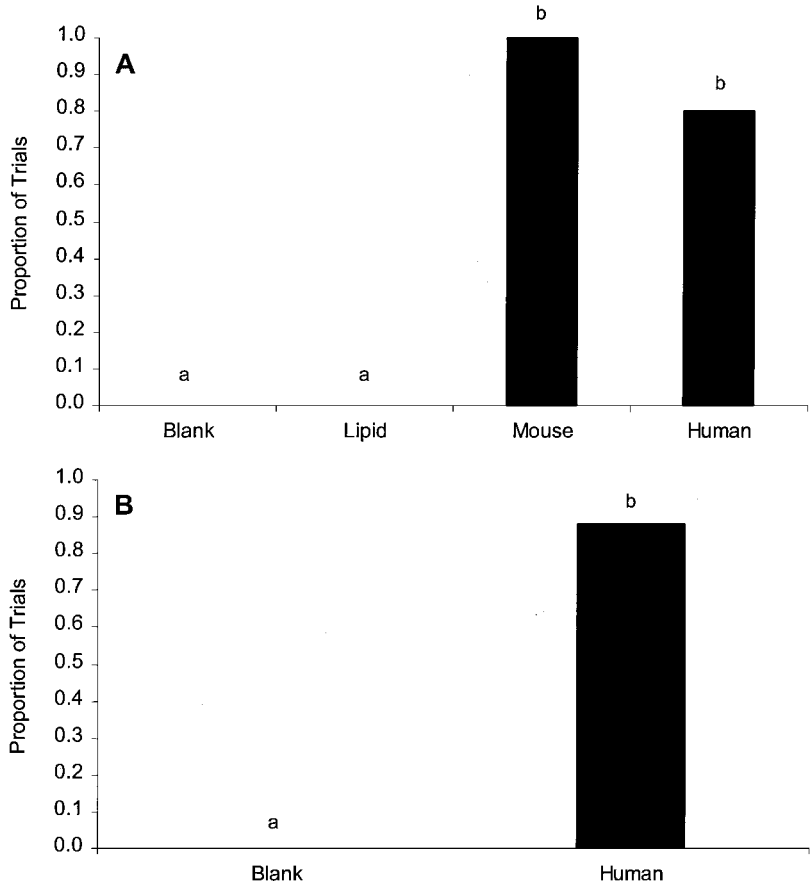


FIG. 1. The proportion of trials in which snakes displayed predatory behavior (close contact tongue-flicks or attacks) to the stimuli for (A) captive snakes originally caught on Guam and for (B) wild-caught snakes from Queensland, Australia. Letters indicate statistical significance between experimental treatments.

semiochemicals that release stereotypical feeding behavior even though the snakes could not ingest the person being attacked. It is unclear if snakes would be attracted to homes specifically because of the presence of human odor, or if the snakes may make accidental contact with humans while searching for prey. This is not the first study to document a predatory response to human stimuli; Chiszar et al. (1993) demonstrated that brown tree snakes show a strong predatory response to human postpartum and menstrual blood.

It appears that the compounds that release feeding behavior from human skin and mice are relatively nonvolatile in nature (for example, the experimental

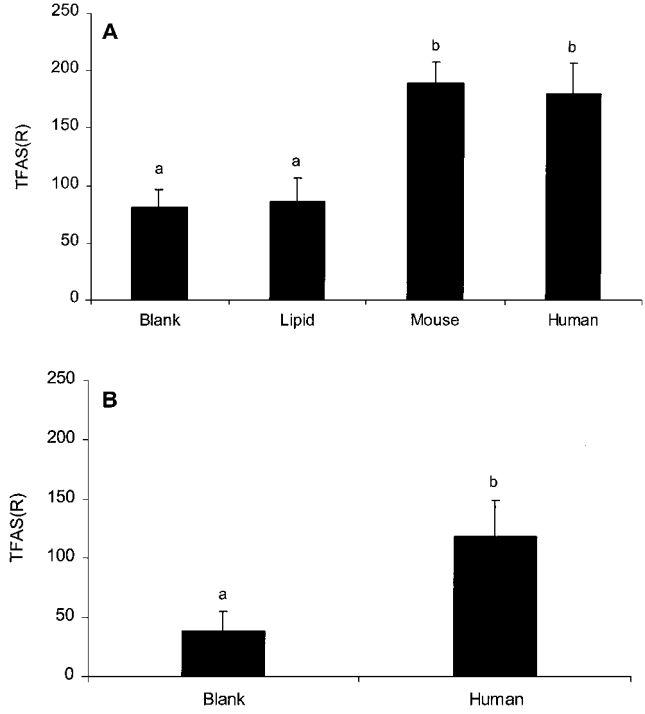


FIG. 2. Tongue-flick attack scores [TFAS(R)] displayed by focal snakes for (A) captive snakes and for (B) wild-caught snakes. Higher TFAS(R) indicate a greater predatory response. Letters indicate statistical significance.

treatments elicited feeding behavior from the snakes several weeks after being stored in a refrigerator). Mammalian skin is covered in a thin film of lipids, composed mainly of sebaceous lipids. Sebaceous glands, associated primarily with hair follicles, secrete a mixture of lipids called sebum onto the skin surface and hair of mammals, while other skin lipids are derived from epidermal cells (Strauss et al., 1991). We hypothesize that this lipid film is a source for the semiochemicals that release feeding behavior in the brown tree snake (Strauss et al., 1991). Human skin lipids are composed of triglycerides (57%), wax esters (26%), squalene (12%), sterol esters (3%), and free sterols (2%) (Greene et al., 1970; Strauss et al., 1991). In mice, the lipid layer is composed mainly of wax diesters (65%), free sterols (13%), sterol esters (10%), and free fatty alcohols (6%) (Wilkinson and Karasek, 1966; Strauss et al., 1991).

The overlap in lipid compounds (or in other types of compounds) between mice and humans might provide an explanation as to why brown tree snakes attempt to prey upon humans. The brown tree snake is known to feed on a diversity of

vertebrate prey, including many species of mammals. We hypothesize that brown tree snakes recognize mammalian prey versus prey of other vertebrate taxa by detecting some skin component common to many mammalian species. Future experiments will examine this in depth; chemical studies will be conducted to determine the chemical structures of compounds that elicit predatory behavior. It would be useful to determine if brown tree snakes respond to specific compounds, or to a broad array of compounds that might be common to their vertebrate prey.

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TRANSPORT OF PHENOLIC COMPOUNDS FROM LEAF SURFACE OF CREOSOTEBUSH AND TARBUSH TO SOIL SURFACE BY PRECIPITATION[1]

P. W. HYDER, E. L. FREDRICKSON,* R. E. ESTELL, and M. E. LUCERO

USDA/ARS, Jornada Experimental Range
Las Cruces, New Mexico 88003, USA

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Abstract—During the last 100 years, many desert grasslands have been replaced by shrublands. One possible mechanism by which shrubs outcompete grasses is through the release of compounds that interfere with neighboring plants. Our objective was to examine the movement of secondary compounds from the leaf surface of creosotebush and tarbush to surrounding soil surfaces via precipitation. Units consisting of a funnel and bottle were used to collect stemflow, throughfall, and interspace precipitation samples from 20 creosotebush (two morphotypes) and 10 tarbush plants during three summer rainfall events in 1998. Precipitation samples were analyzed for total phenolics (both species) and nordihydroguaiaretic acid (creosotebush only). Phenolics were detected in throughfall and stemflow of both species with stemflow containing greater concentrations than throughfall (0.088 and 0.086 mg/ml for stemflow and 0.022 and 0.014 mg/ml for throughfall in creosotebush morphotypes U and V, respectively; 0.044 and 0.006 mg/ml for tarbush stemflow and throughfall, respectively). Nordihydroguaiaretic acid was not found in any precipitation collections. The results show that phenolic compounds produced by creosotebush and tarbush can be transported to the soil surface by precipitation, but whether concentrations are ecologically significant is uncertain. Nordihydroguaiaretic acid was not present in the runoff from creosotebush.

Key Words—*Flourensia cernua*, *Larrea tridentata*, nordihydroguaiaretic acid, total phenolics.

[1]Mention of a trade name, proprietary product or vendor does not constitute a warranty of the product by the USDA or imply its approval to the exclusion of other products or vendors that may also be suitable.

*To whom correspondence should be addressed. E-mail: efredric@nmsu.edu

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INTRODUCTION

Replacement of arid grasslands by shrubs in the southwestern United States has been ongoing since the late 1800s (Buffington and Herbel, 1965; Archer, 1994). Two shrubs that have increased during this transition are creosotebush [*Larrea tridentata* (Sess. & Moc. ex DC.) Cov.] and tarbush (*Flourensia cernua* DC.). Both of these species have extensive root systems (depths of 5 m or more, radial spreads of 1.5–5 m, and surface roots within 10 cm of the soil surface) allowing efficient harvest of water and nutrients compared to grasses associated with these species whose roots rarely exceed 1.5 m in depth (Gibbens and Lenz, 2001). These two species are also well defended against herbivory. The resin on the leaf surface of creosotebush ranges from approximately 8 to 26% of the dry matter, depending on leaf age (Mabry et al., 1977; Rhoades, 1977). The phenolic fraction typically constitutes 80–90% of the resin (Mabry et al., 1977), and this fraction contains mainly phenolic aglycones, with nordihydroguaiaretic acid (NDGA) being the predominant component (Rhoades, 1977). Approximately 5–10% of the dry weight of creosotebush is NDGA (Mabry et al., 1977). Total phenolics in creosotebush ranged from 9.9% of organic matter in leaves collected between July and October (Holechek et al., 1990) to 3.6% in leaves collected in late fall (Hyder et al., 2002). Tarbush also has a resinous leaf surface (8–12% of dry matter) (Estell et al., 1994), with total phenolics ranging from 6.5 to 8.1% for leaves collected in four growth stages over a 3-year period (Estell et al., 1996).

Shmida and Whittaker (1981) concluded that allelopathic effects did not appear to exist for creosotebush based on spatial associations between herbaceous species and creosotebush. Knipe and Herbel (1966) demonstrated that an aqueous extract of creosotebush inhibited germination of black grama [*Bouteloua eriopoda* (Torr.) Torr.] seeds, but did not affect either bush muhly (*Muhlenbergia porteri* Scribn.) or creosotebush seeds. Bush muhly normally grows in close association with creosotebush, occasionally even to the detriment of the creosotebush (Welsh and Beck, 1976). Cox et al. (1983) observed greater leaf lengths and shoot production on grasses grown in soil collected from under creosotebush than in grasses grown in soil from interspaces. Elakovich and Stevens (1985) showed that NDGA inhibited root and hypocotyl growth in eight plant species. We are not aware of any studies regarding the allelopathic potential of compounds from tarbush. However, many phenolic compounds (individually or in mixtures) have been shown to exhibit allelopathic activity toward a number of plant species based on a variety of criteria (Einhellig, 1987; Li et al., 1993; Inderjit, 1996).

de Soyza et al. (1997) described two growth forms of Chihuahuan Desert creosotebush: hemispherical and inverted cone morphotypes (referred to as U and V morphotypes, respectively). These authors suggested the inverted cone shape, which is found among young plants and those in water-limited environments, enhances deep soil water storage through root channeling, while the hemispherical

shape, which is found in established older plants and plants in environments where water stress is less important, enhances soil nutrient development.

Our objectives were to determine if phenolic compounds from creosotebush and tarbush leaves can be transported to the soil surface via precipitation and if concentrations of secondary compounds in runoff differ between creosotebush morphotypes.

METHODS AND MATERIALS

The study site was in the northern Chihuahuan Desert on the USDA/Agricultural Research Service/Jornada Experimental Range approximately 16 km north of Las Cruces, New Mexico, USA. The site consists of an area of sandy loam soil on a 2° east-facing slope. Depth to the first calcic layer is approximately 0.9 m. Gravel and sand at this site were derived from andesites, monzonites, and rhyolites eroded from the nearby Doña Ana Mountains. Elevation at the site is 1322 m. Long-term (1915–1999) mean annual and growing season (July, August, and September) precipitation for the area is 246 and 133 mm, respectively. Long-term mean monthly temperatures for the coldest (January) and warmest (July) months are 6° and 26°C, respectively. Creosotebush is the dominant shrub on this site, with tarbush, mesquite (*Prosopis glandulosa* Torrey), yucca (*Yucca elata* Engelm.), and Mormon tea (*Ephedra trifurca* Torrey) present as minor components. Grass species include burrograss (*Scleropogon brevifolius* Phil.), fluffgrass (*Dasyochloa pulchella* (Kunth) Steudel), black grama, and bush muhly.

During June 1998, 20 creosotebush [10 of each morphotype, as described by de Soyza et al. (1997)] and 10 tarbush were randomly selected by using a point-quarter method. Each plant was equipped with three precipitation collection devices by using methods described by Martinez-Meza and Whitford (1996). One container collected water intercepted by the shrub canopy and channeled down the stems (stemflow). Stems used for collecting stemflow were selected with a leaf cluster unobstructed from above by other branches. The stem collection unit consisted of a polyethylene funnel (11 cm upper diameter with an area of approximately 95 cm^2) glued to the base of a branch with silicone glue. A hole was cut in the funnel and placed such that the funnel completely surrounded the branch. A Nalgene tube attached to the funnel was inserted into a 500-ml Nalgene collection container through a hole drilled in the lid to minimize evaporation and contamination. The tube fit through the hole loosely to allow pressure equalization as the container filled. A second unit collected water passing through the canopy without stem channeling (throughfall), and a third unit (control) collected rain that had not physically encountered a plant (interspaces). Throughfall and interspace collectors consisted of 500-ml collection containers with funnels inserted directly into the container lids. A throughfall collection unit was placed beneath the canopy of each

plant midway between the base and outer edge of the plant, and an interspace unit for each plant was placed an equal distance between it and the nearest neighboring plant. The collection containers were buried half way in the soil to provide stability and minimize exposure to light. Precipitation events occurred on July 22, 25, and 26, 1998, and samples were collected within 30 min of the end of each event. Occasionally, collection units were destroyed by animals or wind, resulting in fewer than 30 samples for a given runoff source/plant species (or morphotype) combination.

Precipitation samples were analyzed for total phenolics and NDGA. Samples were filtered through 0.2-μm PTFE syringe filters (Millex-FG, Millipore Corp., Bedford, Massachusetts, USA). A Waters 2690 separations module coupled to a Waters 486 UV detector (283 nm) equipped with a Symmetry C-18 column (2.1 $\times$ 50 mm, 3.5-μm particle diameter; Waters Corp., Millford, Massachusetts, USA) was used to analyze filtrate for NDGA (100-μl injection size, 65% water–35% HPLC grade acetonitrile mobile phase, flow rate of 0.3 ml/min). Concentrations were quantified with an external standard curve, and either a blank or an NDGA (minimum 90% purity, Sigma Chemical Co., St. Louis, Missouri, USA) standard (ranging from 1 to 100 ng) was injected after every 10 samples to verify calibration. Total phenolics concentration was analyzed using AOAC (1990) procedures (Folin-Denis method, tannic acid as standard). Samples (2-ml aliquots) were analyzed in duplicate on a Milton Roy Spectronic 401 spectrophotometer (760 nm). Statistical analyses were performed using SigmaStat® for Windows, version 2.03 (SPSS, 1997). Statistical procedures used were Kruskal-Wallis one-way ANOVA on ranks with Dunn's method to test all pairwise multiple comparisons.

RESULTS

Total phenolics were detected in both stemflow and throughfall (Figure 1), with greater concentrations in stemflow than throughfall for both creosotebush morphotypes and tarbush ($P < 0.001$). Total phenolic concentrations were higher in stemflow of creosotebush than tarbush, with no difference between the two morphotypes (Kruskal-Wallis one-way ANOVA on ranks, $Q = 0.175$, $P > 0.05$). Mean Stemflow concentrations were (mean $\pm$ SD) 0.088 ± 0.044 ($N = 26$) and 0.086 ± 0.033 ($N = 29$) mg/ml for the U and V morphotypes, respectively. Total phenolic concentrations in throughfall differed between creosotebush morphotypes (Kruskal-Wallis one way ANOVA on ranks, $Q = 1.088$, $P < 0.05$). Throughfall concentrations were (mean $\pm$ SD) 0.022 ± 0.016 ($N = 26$) and 0.014 ± 0.017 ($N = 26$) mg/ml for the U and V morphotypes, respectively. Tarbush exhibited a similar pattern (Figure 1) of greater total phenolics in stemflow than throughfall. Mean concentrations were 0.044 ± 0.022 ($N = 20$) and 0.006 ± 0.009 ($N = 23$) mg/ml for stemflow and throughfall, respectively. Nordihydroguaiaretic acid was not detected in the creosotebush runoff samples, and does not occur in tarbush.

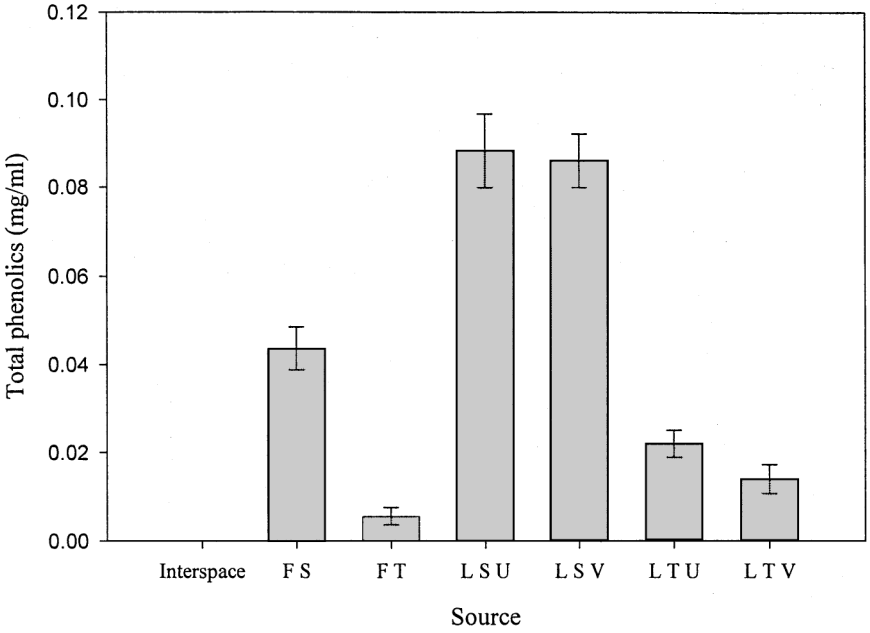


FIG. 1. Total phenolic concentrations (± SEM) in runoff from stemflow (S), throughfall (T), and interspace (I) of tarbush (F) and inverted cone (V) and hemispherical (U) morphotypes of creosotebush (L) during 1998, $N = 5$, 20, 23, 26, 29, and 26, respectively.

DISCUSSION

Creosotebush and tarbush produce phytochemicals with the potential to impact their immediate environment. Phenolic compounds were present in runoff from plant surfaces of both plants, and total phenolic concentrations varied for plant species and source of runoff. Concentration of total phenolics in runoff was greater for creosotebush than tarbush for both sources (approximately two fold for stemflow and three- to four fold for throughfall). Stemflow concentrations were approximately four- to five fold greater than throughfall for both creosotebush morphotypes and nearly an order of magnitude greater than throughfall for tarbush. Concentration of total phenolics in stemflow did not vary between creosotebush morphotypes, but did vary for throughfall (nearly two fold more in U- than V-shaped creosotebush). The hemispherical shape may provide more leaf surface area for precipitation to encounter compared to the inverted cone shape.

Nordihydroguaiaretic acid was not detected in precipitation samples. Potential allelopathic effects of creosotebush are often ascribed to this compound (Knipe and Herbel, 1966; Elakovich and Stevens, 1985). If so, NDGA must reach the soil by

some other presently unidentified route (e.g., leaf litter, root exudates), assuming our results are typical of creosotebush over its geographical and phenological range. Conversely, other phenolic compounds may be responsible for the effects observed in some research, given the wealth of data linking phenolics to allelopathy (Einhellig, 1987; Li et al., 1993; Inderjit, 1996). Lack of movement of NDGA may be due to its hydrophobic nature; this compound is only slightly soluble in water (Botkin and Duisberg, 1949). In prior tests of the effect of creosotebush extracts on seeds of various species, the extracts were obtained by soaking leaves and other tissues in water or Hoagland's solution for extended periods (Dalton, 1962; Knipe and Herbel, 1966; Barbour, 1967). Aqueous extracts (24 hr) of creosotebush contained as much as 150 mg/ml of NDGA (E. L. Fredrickson, unpublished data). Thus, extracts containing high concentrations of NDGA may not be biologically relevant, given the lack of NDGA in runoff observed in this study.

Nevertheless, some phenolic compounds reach the soil surface during precipitation events. Whether they are delivered in quantities sufficient to impact the surrounding environment is unknown. Concentrations of individual phenolics as low as 0.01 mM (Li et al., 1993) and 0.05 mM (Gallet, 1994) have been reported to exhibit allelopathic effects. Total phenolics concentration in our runoff samples would be approximately 0.2–0.4 mM (assuming a mean MW of 200), but this estimate represents a mixture of compounds.

Factors such as intensity, frequency, and duration of rainfall, plant phenology, etc., could affect the amount of secondary compounds delivered to the soil, their distribution (both depth and distance from source), and potential impact on surroundings. For example, Tromble (1988) demonstrated that for creosotebush and tarbush, precipitation events less than 3.6 and 3.0 mm, respectively, would be captured by the canopy and evaporate. Biotic factors may also affect concentrations and persistence of phenolics in the soil. Blum (1998) demonstrated that phenolic acids can have extremely transient lifetimes in soil due to microbial utilization, although Friedman (1987) noted desert ecosystems have properties (low rates of leaching and periodically reduced soil microfauna activity) that may favor persistence of allelopathic compounds.

In conclusion, movement of phenolic compounds from creosotebush and tarbush leaves to the soil surface occurs during precipitation events. Stemflow and throughfall samples from both species contained phenolics, with greatest concentrations in stemflow samples. However, NDGA was not found in any precipitation samples. Actual concentrations of phenolics in soil beneath creosotebush and tarbush are unknown and should be addressed in future work.

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COMPARISON OF DIFFERENT METHODS FOR LIGNIN DETERMINATION AS A BASIS FOR CALIBRATION OF NEAR-INFRARED REFLECTANCE SPECTROSCOPY AND IMPLICATIONS OF LIGNOPROTEINS

KIRSTEN BRINKMANN,[1] LOTHAR BLASCHKE,[2] and ANDREA POLLE[1,*]

[1]*Forstbotanisches Institut, Abtlg I: Forstbotanik und Baumphysiologie*
Georg August-Universität
Büsgenweg 2, 37077 Göttingen, Germany

[2]*Institut für Forstbotanik und Baumphysiologie*
Albert Ludwigs-Universität
Am Flughafen 17, 79085 Freiburg, Germany

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Abstract—Three commonly employed methods for lignin determination, i.e., the thioglycolic acid (TGA), the acetylbromide (AB), and the acid detergent fiber (ADF) method, were compared using leaves and xylem tissue from five species (*Nicotiana tabacum, Populus* x *canescens, Fagus sylvatica. Quercus robur*, and *Picea abies*). In each case, cell walls were isolated before lignin determination. Each of the three methods estimated a different lignin concentration in a given tissue, except for spruce wood. The "lignin" concentration determined with the AB method was strongly dependent on whether or not the cell walls were subjected to alkaline hydrolysis to remove covalently bound aromatic nonligneous components before lignin determination. Lignin concentrations determined in hydrolyzed cell walls of different tissues and species by the AB method showed a good correlation with those obtained by the TGA method and, thus, were convertible. In contrast, gravimetrically estimated ADF lignins did not or only moderately correlate with lignins measured with methods based on the UV absorbance of the solubilized lignin degradation products. Leaves of a given species generally contained higher ADF-lignin concentrations than the corresponding stem tissue. Both ADF and TGA lignin data of beech were used to calibrate near-infrared reflectance spectra (NIRS) for lignin prediction. Both NIRS calibration procedures gave good statistical fits with correlation coefficients close to 1, indicating that TGA and ADF lignin concentrations of beech can be estimated by NIRS with high accuracy. However, the two calibrations were based

* To whom correspondence should be addressed. E-mail: apolle@gwdg.de

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on different empirical terms, showing that TGA and ADF lignins did not share the same physical basis for calibration. C/N analysis revealed the presence of 3.1 and 1.4% nitrogen in ADF lignins of beech leaves and wood, respectively. The major fraction of this nitrogen was recovered in amino acids, which corresponded to 14% and 3% protein in ADF lignins of leaves and wood, respectively. These results show that ADF lignins contain significant concentrations of lignin-bound proteins, which renders this method unsuitable to determine genuine lignin.

Key Words—Acetyl bromide, acid detergent fiber lignin, amino acid, lignoprotein, near infra red reflectance spectroscopy, thioglycolic acid method.

INTRODUCTION

The quantitative determination of lignin is important in ecophysiological research. Lignin is an aromatic heteropolymer predominantly composed of coniferyl alcohol monomers in gymnosperms and of coniferyl and sinapyl alcohol in angiosperms and contains additional *p*-coumaryl alcohol units in grasses (Lewis and Yamamoto, 1990). The subunits are connected by ether (C—O—C) and C—C bonds. Ether linkages dominate, in particular β—O—4 bonds (>50% of the linkages in soft- and hardwoods) (Sjöström, 1993). The quantification of lignin is difficult not only because of its varying monomeric composition but also because lignins are covalently linked with cell wall carbohydrates, proteins, phenolics, or other compounds. These compounds may interfere with determination of lignins leading to over- or underestimation (Monties, 1989).

For determination of lignins, two different principles have commonly been employed: (1) gravimetric methods, which rely on the isolation of lignin or residual cell wall material [e.g., Klason lignin, acid detergent fiber lignin (ADF) (van Soest, 1963; Allen, 1989)] and (2) spectrophotometric methods, which are based on the decomposition of lignins into soluble degradation products and the determination of their absorbance in the UV [e.g., acetyl bromide method (AB), thioglycolic acid method (TGA) (Morrison, 1972; Bruce and West, 1989)]. Both strategies are time-consuming because they require lengthy wet-chemical sample preparation. For routine analysis of large sample numbers high through-put methods are needed. In this respect the use of near-infrared reflectance spectroscopy (NIRS), which takes advantage of the reflectance of chemical bonds in the near infrared, is promising. Near-infrared radiation is absorbed by various chemical linkages such as C—H, O—H, N—H, C=O, C—C, etc., and results in bending, stretching, and twisting of these bonds, leading to characteristic reflectance patterns. NIRS can be applied to milled dry samples without further sample preparation and can be used to determine a range of major chemical components (Foley et al., 1998). NIRS has obtained a certified status to measure moisture, crude protein, and acid detergent fiber content in

forages (AOAC, 1990). It has also been adopted to determine lignin contents, for example, in wood pulp (Easty et al., 1990) and in leaves or leaf litter (Wessman et al., 1988; McLellan et al., 1991; Joffre et al., 1992; Gillon et al., 1993; Bolster et al., 1996).

Cross-comparisons of different methods for lignin determinations are sparse. To date, NIRS methods for lignin determination have been calibrated with Klason or ADF lignins but not with spectroscopic methods exploiting the UV absorbance of soluble lignin degradation products. The goal of the present study was a systematic comparison of three commonly used methods for lignin determination across different species and the application of a gravimetric and spectroscopic method for the calibration of NIRS for lignin determination in different tissues of beech. Because of large differences found between gravimetric and spectroscopic methods, ADF lignins were subjected to C/N and amino acid analysis. The present paper reports for the first time the composition and concentrations of amino acids bound to beech leaf and wood lignins.

METHODS AND MATERIALS

Plant Materials. Stem wood and leaves of field-grown trees (*Picea abies, Fagus sylvatica, Quercus robus trees)* and leaves and debarked stems of greenhouse-grown young plants of *Fagus sylvatica*, *Populus* x *canescens* (hybrid of *P. tremula* x *alba*, clonal material) and of *Nicotiana tabacum* (9-week-old plants) were used for calibration studies. Stem wood from mature 110-year-old dominant beech trees was collected 1.3 m above ground immediately after felling at the field site at Solling (45 m above sea level). Leaves and wood for amino acid analysis in ADF lignins (see below) was collected from about 15-year-old beech trees at the end of August close to the institute (Fuchslöcher). The materials were cut into small pieces, dried at 60 or 70°C (tobacco at 40°C), and were ground to a fine powder (<0.08 mm) in a ball mill (Mikrodismembrator U, Braun, Melsungen, Germany).

Isolation of Structural Biomass for Lignin Analysis. Dry ground plant powder (200 mg) was suspended in 20 ml of washing buffer (100 mM K_2HPO_4/KH_2PO_4, pH 7.8, 0.5% Triton X-100), slowly stirred for 30 min at room temperature, and centrifuged (20 min, 5500g, swing rotor). The pellet was resuspended in washing buffer and washed as above. Subsequently, the pellet was washed 4 times (30 min) in 100% MeOH. The resulting pellet consisted mainly of cell walls, i.e., structural biomass (SBM). The SBM pellet was dried (12 hr at 80°C), weighed, and used for lignin analysis by the thioglycolate method (Bruce and West, 1989).

Alternatively, 400 mg of dry plant powder were suspended in 10 ml of 80% MeOH, stirred for 1 hr at room temperature, and centrifuged at 5500g (20 min). The

pellet was subjected to the following washing steps: 1× (1 M NaCl, 0.5% Triton X-100), 2 × distilled water, 2 × 100% MeOH, and 2 × 100% acetone (30 min each). The resulting AB pellet was used for lignin analysis by the acetylbromide method (Morrison, 1972). The relative weight of the AB pellet (grams of structural biomass per gram of initial dry weight) was slightly lower ($\approx -5\%$) than the weight of TGA pellets. To get rid of esterified phenolics, which interfere with lignin determination by the acetylbromide method, the AB pellet was subjected to alkaline hydrolysis under N_2 (2 M NaOH for 1 hr). The hydrolyzed pellet was washed twice with distilled water and dried at 80°C overnight. The dry pellet was weighed.

Lignin Analyses

TGA Method. Aliquots of 1–2 mg of SBM pellet (3 replicates per individual sample) were weighed into Eppendorf tubes and mixed with 1.5 ml of 2 N HCl and 0.3 ml thiogylcolic acid (adapted after Bruce and West, 1989). Samples were incubated at 95°C for 4 hr and repeatedly mixed. Samples were rapidly cooled on ice and centrifuged for 10 min at 15,000*g* (desk top centrifuge). The supernatant was discarded. Pellets were washed 3-times with distilled water (1 ml). Thereafter, pellets were incubated with 1 ml of 0.5 N NaOH for 18 hr on a shaker at room temperature. The suspension was centrifuged for 10 min at 15,000*g*. The supernatant was carefully transferred into a 2-ml Eppendorf tube. The pellet was resuspended in 0.5 ml 0.5 N NaOH, vigorously mixed, and centrifuged. The resulting supernatant was combined with the first alkaline supernatant and mixed with 0.3 ml conc. HCl. Samples were incubated for 4 hr at 4°C to precipitate the lignothioglycolate derivates. The samples were centrifuged, the supernatant discarded, and the pellet solubilized in 1 ml of 0.5 N NaOH. Absorbance of the resulting solution was measured at 280 nm. Calibration curves were generated by subjecting increasing amounts of 0.5–2.5 mg of commercial lignin (alkaline spruce lignin, Aldrich) to the same procedure. The extinction coefficient of alkaline spruce lignin was $\dot{\varepsilon} = 8.70$ l/g × cm. For comparison, the ADF lignin from beech yielded an extinction coefficient $\dot{\varepsilon} =$ 7.17 l/g × cm.

AB Method. Lignin was determined by the acetylbromide method adapted from Morrison (1972). For this purpose, aliquots of about 2.5 mg of hydrolyzed AB pellet (3 replicates) were mixed with 250 μl of 25% acetylbromide (v/v in glacial acetic acid) and incubated for 30 min at 70°C. Samples were rapidly cooled on ice, mixed with 250 μl of 2 N NaOH, and centrifuged for 5 min at 15,000*g*. An aliquot of the supernatant (250 μl) was mixed with 5 μl of 15 N NH_4OH and 2495 μl of glacial acetic acid. The absorbance of the solution was determined at 280 nm. Calibration curves were generated by subjecting increasing amounts of 0.5–2.5 mg of commercial lignin (alkaline spruce lignin, Aldrich) to

the same procedure. The extinction coefficient of alkaline spruce lignin was $\dot{\varepsilon} = 8.4\ \mathrm{l/g} \times \mathrm{cm}$.

ADF Method. For the gravimetric method of lignin determination (after Van Soest, 1963; Rowland and Roberts, 1994) 1 g of milled dry plant material was weighed (W1) into a 250-ml Erlenmeyer flask and boiled for 1 hr in 100 ml cetyltrimethyl ammonium bromide solution (1 g cetyltrimethyl ammonium bromide in 100 ml of 0.5 M H_2SO_4) under continuous stirring. A drop of octan-2-ol was added as an antifoam agent. The solution was filtered over an ignited and preweighed sinter (Robu-Glas, No. 2, 16–40 μm, Jena) and washed 3-times with 50 ml of hot distilled water. The filtrate was washed with acetone until further decoloration was not observed. The filtrate was dried for 2 hr at 105°C. About 10 ml of cool 72% H_2SO_4 (15°C) was added to the cooled sinter and mixed with the filtrate. Draining acid was refilled keeping the mixture for 3 hr in 72% H_2SO_4. Thereafter, the acid was filtered off under vacuum, and the residue was washed with hot distilled water until it was acid-free. The sinter was dried at 105°C for 2 hr, cooled, and weighed (W2). The sinter was ignited at 500°C for 2 hr, cooled, and weighed to determine ash content of the residue (W3). Lignin (%) was calculated as $(\mathrm{W2} - \mathrm{W3}) \times 100/\mathrm{W1}$.

For the determination of leaf lignins, it was necessary to add a filter aid (Celite 545, Baker, Deventer, The Netherlands).

NIRS Method. Ground, dry plant powder was scanned with monochromatic light at 2-nm intervals in a near-infrared reflectance spectrometer (NIRS system 6500, Foss, Hamburg, Germany) in the range of 400–2500 nm in two replicates per sample. All reflectance values are relative to a ceramic standard. Software used for data collection and data analysis was WINISI-II-Software systems (Foss). Each reflectance spectrum (R) consisted of 1050 data points and was automatically converted to apparent absorbance $[\mathrm{A} = \log(1/R)]$ prior to storing the spectral values. The inset in Figure 1 shows a typical sample spectrum. To minimize scattering and to accentuate fine-scale spectral features, the spectra were math transformed to the second derivative (Figure 1). Calibrations were developed for ADF lignins and TGA lignins using the built-in software package (WINISI-II-Software systems, Foss). The calibration equations were calculated using the modified partial least square regression method (MPLS) (as described by Bolster et al., 1996; Gillon et al., 1999). This calibration type selects wavelengths over the whole spectrum strictly on the basis of statistical correlation (principle component analysis) and is not based on preselected wavelengths. To avoid overfitting, cross-validation was used to obtain the optimal number of terms in the calibration. For this purpose, 5/6 of the samples were used to develop the model and 1/6 for the prediction. This was repeated 6 times and all the residuals of the 6 predictions were pooled to provide a standard error of cross-validation (SECV) of independent samples. The final model was then calculated to obtain the standard error of calibration

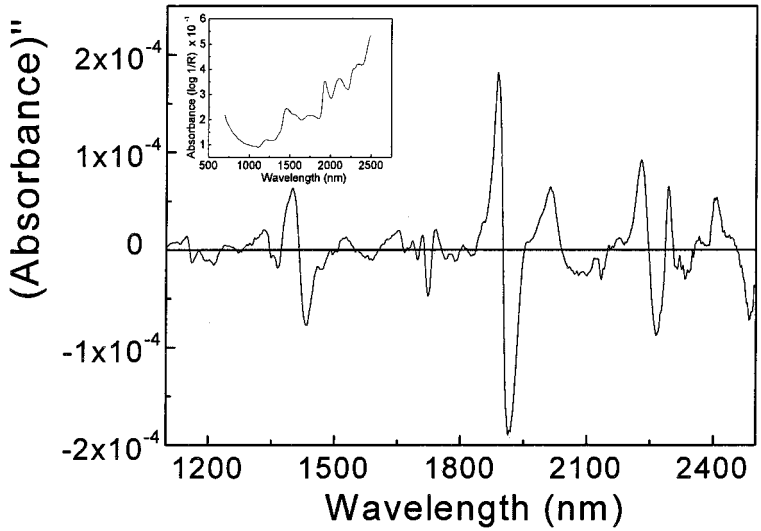


FIG. 1. Near-infrared reflectance spectrum of beech stem wood after log $1/R$ transformation and math treatment (2nd derivative). The original spectrum is shown in the inset. The spectrum is the mean of four samples measured at 2-nm gap width.

(SEC). For each calibration, different mathematical algorithms of the wavelength scans were compared. The math treatment, that gave the lowest SECV was selected. It has been reported that the whole-spectrum calibration mode provides the best fit to the data, but usually the calculated correlation coefficients of individual wavelengths can not be explained in a meaningful way (Bolster et al., 1996). Individual wavelengths that affected calibration were obtained by factor loading. For ADF and TGA lignin calibrations, 6 and 8 factors, respectively, have been obtained representing the wavelengths and biophysical properties indicated in Table 1.

Amino Acid Analysis. For the analysis of amino acids, 30 mg of ADF lignins were incubated in 0.5 ml of 5 N HCl for 24 hr at 105°C. After centrifugation (10 min, desktop centrifuge), the acidic extract was diluted (1:1000), and 40 μl of the dilution were evaporated, subsequently dabsylated, and analyzed by HPLC as described previously (Chang et al., 1992). Instead of dry wood or leaves, ADF lignins were also isolated from fresh plant materials in a washing buffer also containing soluble 2% polyvinylpyrolidine (PVP K-30).

Statistical Analysis. Where appropriate, the data were analyzed by linear regression. The analytical error of individual samples was generally below 5%. Data in the tables and figures indicate means of individual plants ($N = 5$ to 10, $\pm$SD).

TABLE 1. FACTORS OBTAINED BY PRINCIPLE COMPONENT ANALYSIS GIVING BEST FIT NEAR INFRARED (NIR) CALIBRATIONS AND CORRELATED WAVELENGTHS[a]

Wavelength (nm)														
ADF-Lignin Factor						TGA-Lignin Factor								
1	2	3	4	5	6	1	2	3	4	5	6	7	8	Chemical Property
								736						—
			760	760										—
1324														—
	1372				1372				1364					C−H CB−CH_3
				1404			1404							C−H CB−CH_3
		1428	1428							1436	1444	1436		C−H,CB−CH_3, CB-aromatic OH-stretch, 1st OT starch
1652														—
		1676			1676				1676	1668		1668		CH stretch, 1st OT-aromatic
		1724	1724	1724	1724				1724	1724				C−O oil, C−H-stretch 1st OT CH_2
1860	1852					1868								—
				1908			1924	1924		1916	1916	1908		O−H stretch, 1st OT P−OH C=O stretch, 2nd OT CONH O−H stretch
2012						2004							1996	N−H stretch, N−H bend, CB-urea
	2036		2052											C=O stretch 2nd OT urea, N−H amideII, CB−$CONH_2$ N−H stretch amideI, CB protein
				2116							2124			O−H bend C−O stretch, CB starch, C−O−O stretch 3rd OT starch or cellulose
								2140	2140	2140			2148	C−H stretch C=O, CB oil
					2172									C−H stretch, C−H deform, CB H−C=C−H, N−H bend 2nd protein, CH stretch C=O, CB protein CH stretch amide III CB protein
							2188			2140				
2220	2220													C−H stretch C=O, CB CHO
				2244		2212								—
		2260	2260					2252	2260	2252	2268	2252		O−H stretch C−O CB cellulose
2308				2308	2308	2308	2308	2308					2300	C−H bend 2nd OT protein, C−H bend 2nd OT oil
													2340	—
				2388	2388			2396	2380	2380	2380	2380		C−H stretch, C−C oil

[a] The wavelengths were grouped at a step width of 16 nm because the math treatment was based on an interval of 8. The right column of the table indicates the corresponding chemical structures. OT = overtone, CB = carbon bond.

RESULTS AND DISCUSSION

Comparison of Lignin Concentrations Determined by Different Methods and across Different Species. To find out the suitability of different methods for lignin comparisons across species and for different plant tissues, leaves and xylem tissue of four woody and one herbaceous species (*Picea abies*, *Quercus robur*, *Fagus sylvatica*, *Populus* × *canescens*, and *Nicotiana tabacum*, respectively) were analyzed. Three different protocols were used: the TGA method, the AB method before and after alkaline hydrolysis, and the ADF method. Both the AB and TGA methods rely on the solubilization of lignin, in one case due to acetylation, in the other case because of formation of thiolignogylcolic acids. The absorbance of the resulting aromatic compounds was determined in the UV and calibrated with alkaline spruce lignin to determine lignin concentrations. The ADF method is a gravimetric method. The rationale is to remove hemicelluloses and proteins by washing plant materials in acid containing detergents (Rowland and Roberts, 1994). Subsequent treatment with 72% H_2SO_4 results in degradation of cellulose, yielding a residual fraction thought to be mainly composed of lignins. The weight of this fraction corrected for ash contents was taken as "ADF lignin."

The TGA method generally gave lower lignin concentrations in the tissues than the other methods tested (Figure 2). Such differences between different methods for lignin determination have also been reported by others. For example, Pillonel et al. (1991) determined about 7-fold lower lignin concentrations in maize leaves with the TGA method than with the Klason method, which is similar to the ADF method. Surprisingly, we found that leaves contained higher "ADF lignin" contents than stem wood, with the exception of spruce wood (Figures 2A,B). Variable differences were found between the ADF and the TGA methods, e.g., in tobacco leaves 100% ADF lignin corresponded to only 9% TGA lignin, whereas spruce wood gave relatively consistent results (Figure 2). The lignin concentration of dry stem spruce wood accounted for 25% and 27% using the TGA and ADF methods, respectively, and, thus, were in the range of previously reported data [28.2% by Fengel and Wegener (1989) 27.4% by Sjöström (1993)]. The AB method yielded a slightly lower lignin concentration of 21%.

The "lignin" concentrations determined by the AB method were dependent on whether the samples had been subjected to alkaline hydrolysis or not (Figure 2). Alkaline hydrolysis releases phenolics that are covalently bound to lignins (Strack et al., 1989). Since these compounds also have a strong absorption in the UV, they will erroneously be determined as lignins if not removed prior to the acetylbromide treatment. However, alkaline hydrolysis may also result in a small nonspecific loss of lignins (Akin et al., 1995). Except for spruce wood, all other species and tissues analyzed contained significant amounts of aromatic components, which were soluble by alkaline hydrolysis (Figures 2A,B). Leaves generally contained

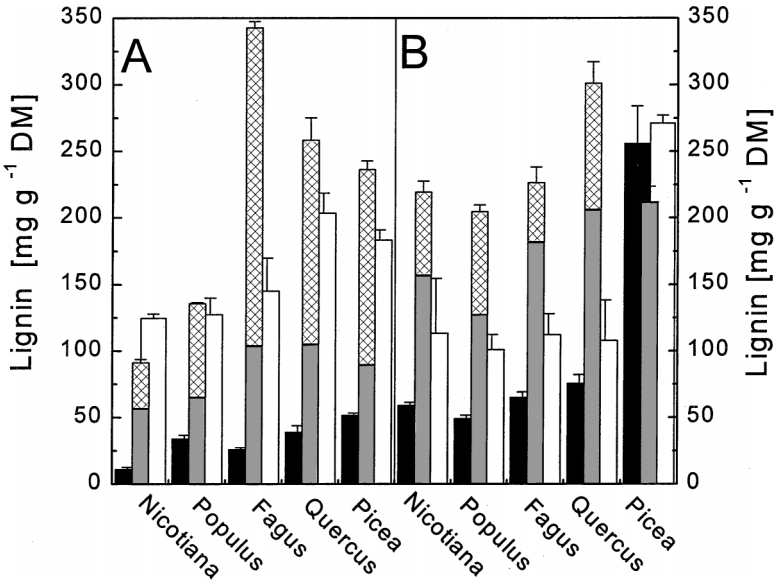


FIG. 2. Lignin concentrations in leaves (A) and stem wood (B) of different plant species. Lignin was determined with the TGA method (black bars), with the AB method before (hatched bars + gray bar) and after alkaline hydrolysis (gray bars), and as ADF lignin (white bars). Bars indicate means of 6 replicates ± SD. DM = dry mass.

higher concentrations of these components than wood (Figures 2A,B). This fraction was particularly high in beech leaves.

To determine whether the methods for lignin determination were convertible, regression analyses were performed. A significant correlation was found between the TGA and the AB methods (Figure 3A, $R = 0.888$, $P < 0.001$), although the absolute lignin concentrations determined by the two methods differed by a factor of 2.58. No correlation was found, however, between lignin determined by the TGA and the AB methods before alkaline hydrolysis ($R = 0.432$, $P = 0.245$, not shown). Furthermore no significant correlation was found for lignin concentrations obtained with the TGA and ADF methods in different species (Figure 3B, $R = -0.274$, $P = 0.474$). However, a moderate correlation existed between TGA and ADF lignins, if different tissues of one species, i.e., beech, were analyzed (Figure 3B, $R = 0.690$, $P = 0.001$).

The results indicate that both UV methods can be used for comparisons of relative lignin concentrations across different species and tissues. However, to obtain convertible estimates for lignin by the two UV methods, it is neccessary to remove cell wall-bound aromatic components by alkaline hydrolysis before the solubilization of lignin by acetyl bromide. Such a step is not necessary for the

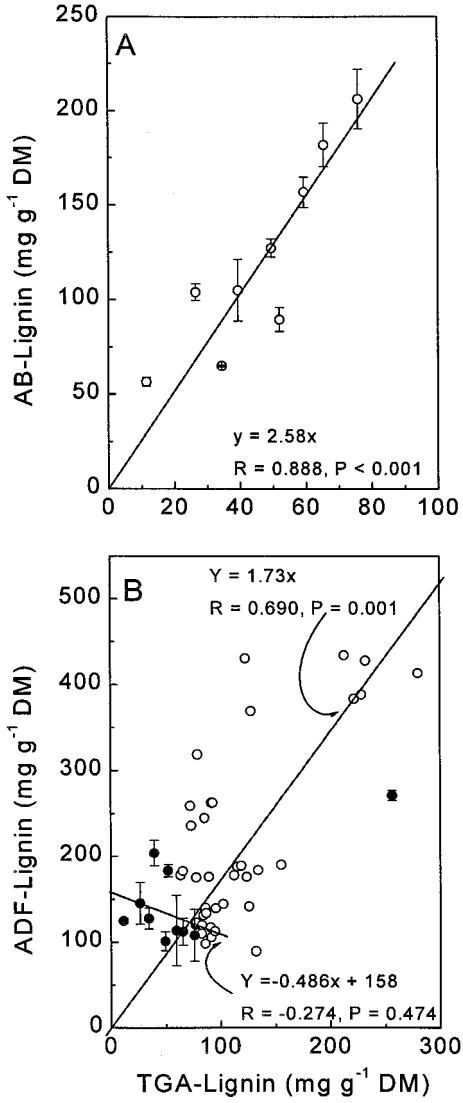


FIG. 3. Correlation analysis between TGA and AB lignin across species (A) and between TGA and ADF lignins (B) across species (full circles) and for different beech tissues (open circles). DM = dry mass.

determination of lignins with the TGA method because thioglycolic acid attacks specifically ether bonds in lignin, which link the monomers at the propanoid unit. The thioglycolic adduct is subsequently separated from other soluble components before measuring its absorbance in the UV. Thus, the TGA method mainly titrates β—O—4-bonds, and, therefore, is specific for lignins. The formation of thiolignoglycolate derivatives is considered a criterion for "genuine" lignin (Freudenberg, 1968). However, because of this specificity for certain properties of lignins, this method is also likely to underestimate the overall lignin concentration. In contrast to the TGA method, the AB method is likely to overestimate the lignin concentration slightly because incubation of cell walls with acetylbromide may lead to some oxidative degradation of structural polysaccharides, especially of xylans, resulting in increased absorbance at 280 nm, the wavelength used to determine lignin (Hatfield et al., 1999). However, it should be pointed out that we used short incubation times with acetyl bromide (30 min), which according to Hatfield et al. (1999) did not yet yield significant unspecific absorbance.

An important result was that the UV methods for lignin determination were not convertible with the gravimetric ADF method for comparisons across species. ADF lignins have been defined operationally as that organic material that can not be digested by 72% sulfuric acid.

Calibration of NIRS for Determination of Beech Lignin. In the literature, ADF lignins, which are prepared in a similar manner as Klason lignins (Rowland and Roberts, 1999), have been employed successfully to develop algorithms for lignin calibration curves by NIRS despite the undefined chemical nature of these "lignins" (e.g., McLellan et al., 1991; Gillon et al., 1993; Bolster et al., 1996). In the present study, we used ADF and TGA lignin data for calibration of NIRS. To avoid problems potentially arising from differences between species, ADF and TGA-lignin concentrations were determined in different tissues of one species, i.e., in leaves, stem wood, and coarse and fine roots of beech (cf. Figure 3B). A typical example of a NIR spectrum is shown in Figure 1 (inset). Based on such spectra, calibrations were developed based on both TGA and ADF lignin determinations (Figure 4). The correlation coefficients of the resulting relationship between measured and predicted values, especially the one determined on TGA-based calibrations, were high (Table 2). Expressed on a percentage basis, the SECV—a measure for the accuracy of the method—was about 1.4 for both calibration procedures (Table 2) and, thus, about 2 times lower than the SECV reported by Joffree et al. (1992). However, in preceding studies, lignins from different plant sources or from leaf litter in different stages of decay have been used (McLellan et al., 1991; Gillon et al., 1993; Bolster et al., 1996). Under these conditions, changes in the matrix structures, which may affect reflectance patterns, can not be excluded and might have caused a slightly higher scattering of the data than in the present study. NIR spectroscopy of lignin has also been successfully applied in pulp analysis (Wallbäcks et al., 1991).

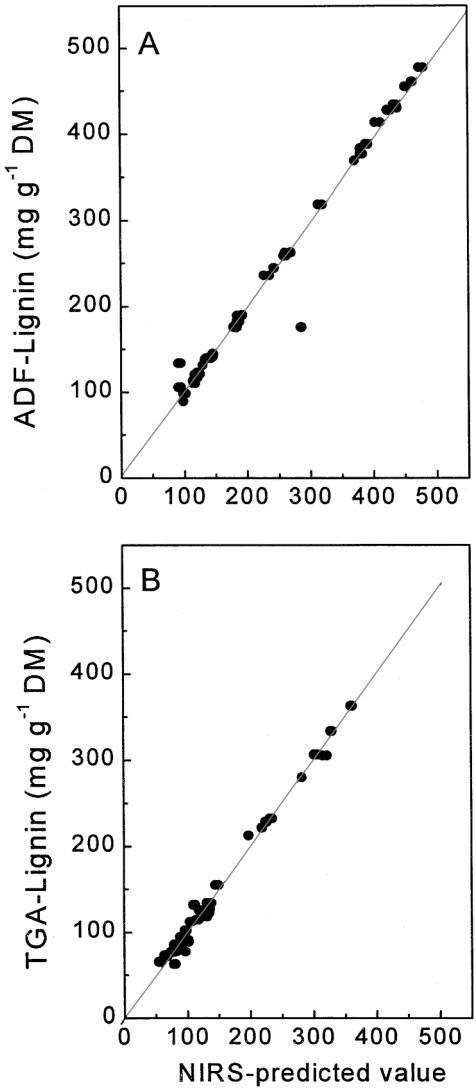


FIG. 4. Relationship between NIRS predicted lignin concentrations and ADF lignin (A) and TGA lignin (B) from different beech tissues. DM = dry mass.

The calibration curves produced here were used to predict native lignin concentrations in stem wood of mature beech trees. Employing the ADF calibration, we obtained a mean lignin concentration of 24.9% (Table 2), which is almost identical with figures published for beech wood previously [22.8% and 24.4%

TABLE 2. PREDICTION STATISTICS OF LIGNIN DETERMINED BY TGA AND ADF METHODS, DETERMINED OVER WHOLE SPECTRAL RANGE (400–2500 nm) AND DETERMINATION OF LIGNINS IN STEM WOOD OF MATURE BEECH TREES WITH NIRS[a]

Lignin	*N*	Mean	Range	SD	SEC	R^2	SECV	MT	Predicted lignin (mg/g)
TGA	92	130.3	63.4–363.1	78.3	8.06	0.989	13.8	2,4,4	87.2 ± 11.5 (n = 12)
ADF	92	231.8	89.6–477.7	119.2	7.56	0.996	13.3	2,4,4	249.6 ± 20.2 (n = 12)

[a] MT = mathematical transformation of spectral data: the first number is the order of the derivative function, the second the segment length of the data points over which the derivative was taken, and the third is the segment length over which the data were smoothed. SEC = standard error of calibration, SECV standard error of cross-calibration.

by Fengel and Wegener (1989) 24.8% by Sjöström (1993)]. Employing the TGA calibration, lignin concentrations of less than 10% were predicted for beech stem wood (Table 2). These values appear low as compared with ADF lignins. In a subset of the stem wood samples, ADF and TGA lignins were determined for validation. The deviation between NIRS-predicted values for ADF lignins and ADF-measured values was 1.3%, and that for NIRS-predicted and measured TGA values 2.7%.

These results clearly show that NIRS calibration curves based on different tissues (leaves, wood, fine and coarse roots) can predict lignin values for beech with high accuracy. However, the absolute lignin concentrations obtained in this way depend on the method chosen for calibration. Both methods employed here show differences in the NIRS calibration, which can not simply be explained by different scaling factors between the two methods because of differences in (1) the range of the apparent lignin concentrations determined by two methods (6.3–36.9% for the TGA, 8.9–47.7% for the ADF method, Table 2) and (2) the distribution of the data over the calibration curve (relatively homogenous distribution of ADF-data, relative clumping of TGA-data). Since the measurements were performed on the same set of samples, it is clear that ADF and TGA lignins must have a different chemical basis. This can also be inferred from results of principle component analysis, because the factors, which have been developed for NIR calibration only on statistical principles and not by *a priori* selection of wavelengths typical for lignin, revealed that different ranges of wavelengths contributed to ADF and TGA lignins, respectively (Table 1). In addition to some overlap, e.g., of wavelengths where aromatic compounds reflect (1428–1444 nm, and 1668–1678 nm), the calibration of ADF lignins was also affected by wavelengths, where amines or amino acids reflect (2036–2055 nm, 2244 nm) and that of TGA lignin by reflection of $\cdot NC{=}CH$ (2140 nm).

Presence of Ligno Proteins in ADF-Lignins. Recently, it has been reported that gravimetrically prepared lignins, e.g., by the Klason or the ADF method, containe nitrogen (Rowland and Roberts, 1999, Dyckmans et al., 2002). To find out

TABLE 3. CARBON AND NITROGEN IN DIFFERENT FRACTIONS OF LEAVES AND WOOD OF BEECH (*Fagus sylvativa*)[a]

	DM	ADF lignin	NLCW	Soluble matter
Leaves				
Mass (mg)	1000	99 ± 15	367 ± 55	534 ± 80
Carbon (mg/g DM)	472 ± 1	52 ± 0.3	148 ± 1	272 ± 0.9
Carbon (%)	47.2	52.5	40.3	50.9
Nitrogen (mg/g DM)	17.8 ± 0.1	3.06 ± 0.04	4.19 ± 0.07	10.55 ± 0.07
Nitrogen (%)	1.78	3.09	1.14	1.97
C/N	27	17	35	26
Wood				
Mass (mg)	1000	103 ± 7	590 ± 43	307 ± 22
Carbon (mg/g DM)	468 ± 1	51 ± 1	292 ± 6	124 ± 0.3
Carbon (%)	46.8	49.5	49.5	40.3
Nitrogen (mg/g DM)	3.33 ± 0.05	1.36 ± 0.03	1.65 ± 0.05	0.31 ± 0.004
Nitrogen (%)	0.33	1.34	0.28	0.10
C/N	142	37	176	403

Note: NLCW = nonligneous cell walls.
[a] For further explanation, see text. ($N = 5$, ±SD).

the chemical nature of this nitrogen and to assess the contribution of this fraction to the mass of ADF lignins, C/N and amino acid analyses were performed with beech leaf and wood preparations (Table 3). The following fractions were distinguished: whole-tissue dry matter, ADF lignins, nonligneous cell walls (= SBM ADF lignins), and soluble matter (= whole-tissue dry matter—SBM) (Table 3). The interesting result was that the relative carbon concentration of the different fractions showed only little fluctuation, whereas nitrogen was relatively enriched in ADF lignins compared to nonligneous cell walls or soluble components (Table 3). ADF lignins of leaves contained 11% of the foliar carbon and 17% of the foliar nitrogen, and those of wood contained 11% of the carbon and 41% of the nitrogen. Overall, our data show that about 40% of the leaf nitrogen and 90% of the wood nitrogen were bound to the cell wall fraction. Within this fraction nitrogen was relatively enriched in lignins as indicated by low C/N ratios of ADF lignins as compared with the C/N ratios of other fractions (Table 3). Since the above figures were obtained with preparations of fresh plant materials after extraction of soluble compounds with detergents in the presence of phenol scavengers, unspecific covalent binding of soluble proteins to lignins during extraction is unlikely. If dry plant material is used to isolate SBM and ADF lignins, the concentration of bound nitrogen is about 35% higher (data not shown).

Since cell wall carbohydrates may bind various functional groups forming, e.g., glucosamines, the nature and potential contribution of nitrogen to the mass of ADF lignins is not clear *a priori*. To find out whether the observed nitrogen was proteinaceous, ADF lignins were digested for amino acid analysis. We found

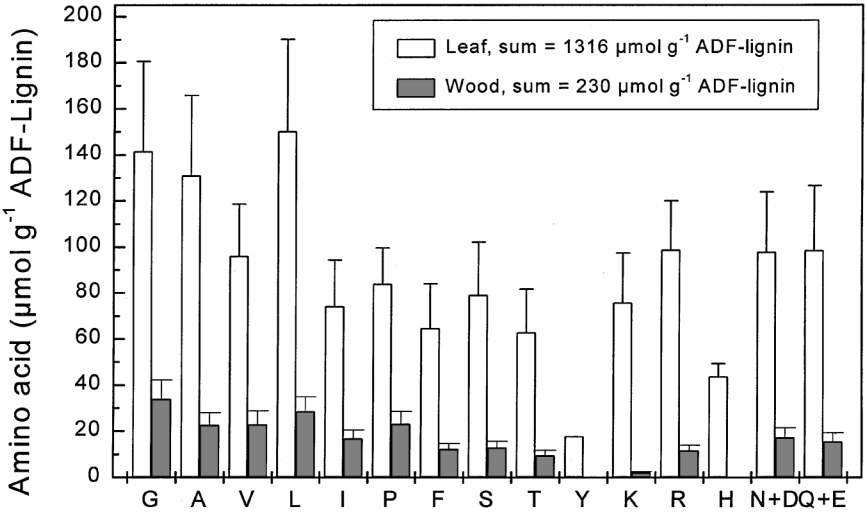


FIG. 5. Amino acids in ADF lignins from wood and leaves of beech (*Fagus sylvatica*) ($N = 5, \pm$SD). The amino acids were indicated with their biochemical one-letter-code: G = glycine, A = alanine, V = valine, L = leucine, I = isoleucine, P = proline, F = phenylalanine, S = serine, T = threonine, Y = tyrosine, K = lysine, R = arginine, H = histidine, N + D = asparagine + aspartate, Q + E = glutamine + glutamate.

that leaf lignins contained approximately a 5.7-fold higher concentration of amino acids than wood lignins (Figure 5). The amino acids covered the known spectrum of proteinaceous amino acids. Sulfur-containing amino acids (methionine, cysteine) can not be detected, and glutamate and glutamine (Q + E), as well as aspartate and asparagine (N + D), can not be separated by this method. Tryptophan, which occurs only at a low frequency in proteins (1.1%), was not found and may have been below the detection limit.

The amino acid spectra of wood and leaf ADF lignins were compared to find out whether the amino acid composition of putative proteins bound to wood-lignins was similar to that of lignoproteins from leaves. For this purpose, the relative frequency of each individual compound was calculated. The relative frequency of amino acids in leaf ADF lignins was plotted against those of wood ADF lignins. Amino acids occurring with the same abundance in both lignins are expected to appear on a curve with a slope of 1 (Figure. 6). Figure 6 shows that some nonpolar amino acids [proline (P), valine (V), and glycine (G)] were relatively enriched in proteins bound to wood lignins, whereas some polar amino acids, characterized by elevated nitrogen content [arginine (R), lysine (K), and histidine (H)] were more abundant in proteins bound to leaf-lignins than in those from wood. Furthermore, tyrosine (Y), an aromatic amino acid with a hydroxyl group, was also

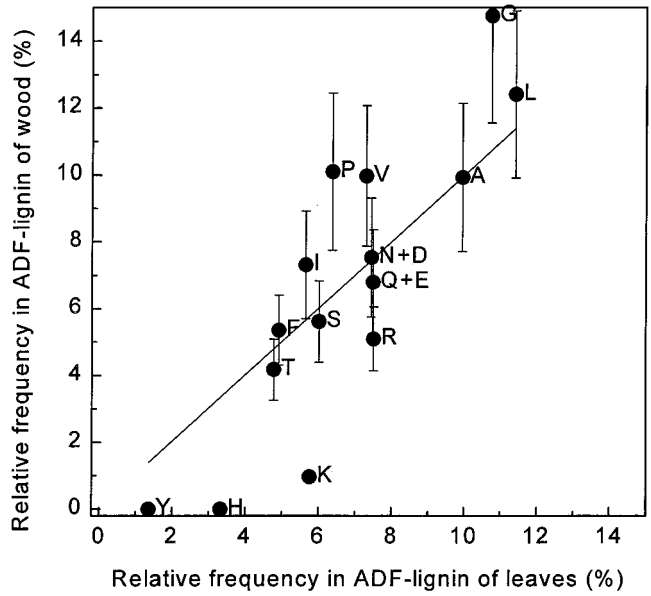


FIG. 6. Relative frequency of amino acids from ADF lignins of wood in relation to the relative frequency of amino acids from ADF lignins of leaves from beech (*Fagus sylvativa*). For wood and leaves, 230 μmol and 1316 μmol amino acid/g ADF lignin, respectively, were set as 100% (data from Figure 4) and used to express the relative frequency of each individual compound. Letters in the figure refer to amino acids using their one-letter-code as in Figure 5.

more abundant in ligno proteins from leaves than in those from wood. The nature and function of these lignoproteins needs to addressed in further studies.

The sum of the amino acids extracted from leaf ADF lignins corresponded to 75% and those from wood ADF lignins to 26% of the total nitrogen found in these fractions. On a mass basis, the amino acids corresponded to 141 mg protein/g ADF lignin in leaves and to 23 mg protein/g ADF lignin in wood. If all nitrogen found in ADF lignins were due to protein, the above figure would increase to 188 mg protein in ADF lignins from leaves, i.e., almost 20% by mass, and to 88 mg protein in ADF lignins from wood, i.e., about 9% by mass.

In summary the comparison of two spectroscopic methods (AB and TGA methods) showed that the amount of lignin determined by its absorbance in the UV may vary significantly depending on the calibration standard and on the pretreatment of the sample. Although the AB method is easy to use and reliable, as pointed out the Rodrigues et al. (1999), one has to be aware that even washed cell walls still contain bound aromatic compounds, which interfere with the lignin determination in a species- and tissue-dependent manner. The TGA method generally

yielded lower lignin concentrations than other methods but was convertible with the AB method and did not require hydrolysis of cell walls before lignin analysis. Both methods can be applied to small samples.

The gravimetric ADF method for lignin determination gave the highest lignin concentrations. However, ADF lignins contained considerable concentrations of bound protein, even though washed cell walls had been used for the preparation. The contribution of proteins to ADF lignins was estimated to be in the range of 2–9% in wood and 14–18% in leaves of beech. These results indicate that the ADF-lignin method will overestimate lignins to different degrees in leaves and wood and, thus, is not suitable to compare concentrations of "genuine" lignin in different tissues. Both ADF and TGA lignins were suitable for calibration of NIRS and predicted the "lignin," i.e., the chemical proper used for calibration, with high accuracy. However, in the case of ADF lignins, the predicted fraction should not be termed lignin but instead, lignoprotein.

It is known that cell walls contain structural protein and, thus, must contain nitrogen (Carpita and Gibeaut, 1993). Furthermore, it has been reported that proteins involved in the synthesis of lignins also become covalently bound during this process (Ros Barcelo, 1997). Still, the fractions of cell wall or lignin-bound nitrogen have not been quantified before. The present study shows for the first time that lignoproteins constitute a significant fraction of nitrogen relative to whole-tissue nitrogen. Despite the apparent abundance of lignoproteins nothing is known about their physiological functions and ecological implications. It is tempting to speculate that this fraction acts as a slow-release fertilizer in forest ecosystems. It will be interesting to study the turnover rate of lignoproteins and their significance in C and N cycles.

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BRUCHINS, PLANT MITOGENS FROM WEEVILS: STRUCTURAL REQUIREMENTS FOR ACTIVITY

JAMES E. OLIVER,[1,*] ROBERT P. DOSS,[2] BRIAN MARQUEZ,[3,4] and E. DAVID DEVILBISS[1]

[1]*USDA, Agricultural Research Service*
Chemicals Affecting Insect Behavior Laboratory, BARC-W
Beltsville, Maryland 20705, USA

[2]*USDA, Agricultural Research Service*
Horticultural Crops Research Unit
Corvallis, Oregon 97330, USA

[3]*College of Pharmacy*
Oregon State University, Corvallis
Oregon 97331, USA

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Abstract—Bruchins are 3-hydroxypropanoate esters of long-chain α,ω-diols from pea weevils and cowpea weevils that have been shown to initiate callus formation on pea pods at extremely low application rates. Synthetic analogs have been prepared and examined to evaluate structural requirements for inducing this unusual neoplastic response. Chain length (optimum length C_{22}–C_{24}) is important, whereas unsaturation within the chain is relatively unimportant. Difunctionality is required for maximum activity, but the α,ω-diols themselves are inactive. Most critical is the ester portion(s) of the molecules; 3-hydroxypropanoate esters are far more active than any analogs examined.

Key Words—Bruchins, mitogens, Bruchidae, 3-hydroxypropanoates.

INTRODUCTION

We recently reported (Doss et al., 2000) the isolation and identification of four compounds, which we have named bruchins, from two species of Bruchidae [pea weevils (*Bruchus pisorum* L.) and cowpea weevils (*Callosobruchus maculatus* F.)] that stimulate rapid proliferation of undifferentiated cells when applied to pea

[4]Current address Wyeth-Ayerst Research, Discovery Analytical Chemistry, Collegeville, Pennsylvania 19426.

[*]To whom correspondence should be addressed. E-mail: oliverj@ba.ars.usda.gov

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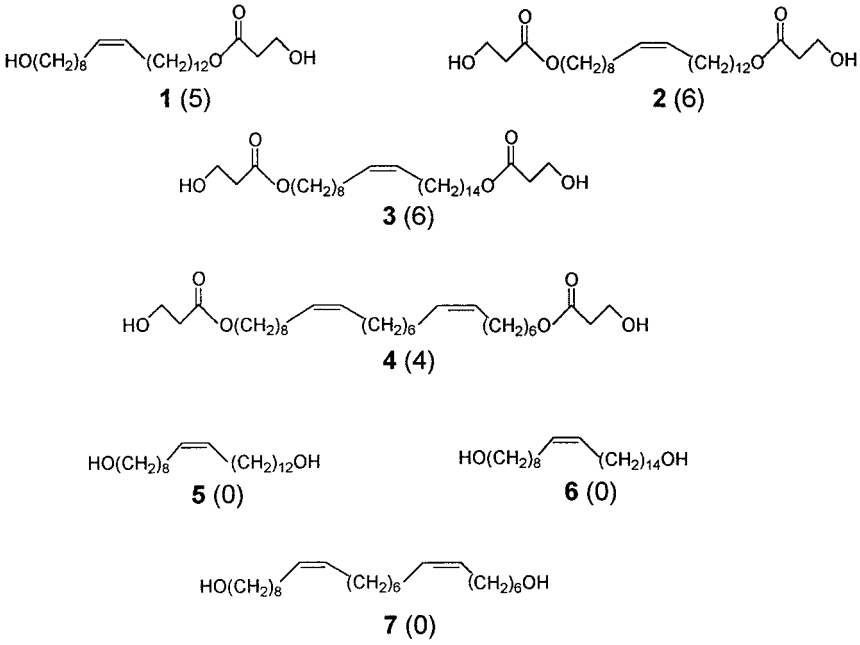


FIG. 1. Naturally occurring bruchins and corresponding α,ω-diols (relative activity in parentheses; see table).

pods (Doss et al., 1995). The work culminating in isolation and identification of these previously unreported compounds was initiated in response to reports that oviposition by pea weevils on pods of pea plants possessing the *neoplastic pod* (*Np*) gene resulted in the formation of neoplastic tissue (callus) beneath the eggs. (Berdnikov et al., 1992). Callus formation, in turn, inhibits larval entry into the pod (Berdnikov et al., 1992; Doss et al., 2000). These neoplasm-inducing compounds, which are mono- and bis-(3-hydroxypropanoate) esters of C_{22} and C_{24} α,ω-diols (**1**–**4**, Figure 1), are unprecedented as plant growth regulators, or for that matter, as natural products, and their remarkable activity (measurable callus formation can result following applications of as little as 0.5 pg of a bruchin) stimulated our interest in what structural features of these compounds contribute to, or are necessary for, this unusual biological activity.

We have described (Oliver et al., 2000) syntheses of the natural bruchins **1**–**4**, and reported that although the mono- and diesters **1** and **2** seemed comparably active in promoting cell division, the corresponding diols **5**–**7** themselves did not stimulate neoplasm formation, indicating that at least one 3-hydroxypropanoate moiety was involved in the growth-triggering response. We describe here a more detailed investigation into the structural requirements for bruchin activity that includes

evaluation of several features of the diol backbone (chain length and rigidity, unsaturation, geometry, structural modification), mono- versus difunctionalization, as well as examination of the effects of a variety of structural modifications in the acid portions of the molecules.

METHODS AND MATERIALS

Because of the large number of compounds discussed in this paper, we are omitting experimental details. Some generalizations concerning experimental procedures and strategies are outlined in the Results and Discussion section and in a previous publication (Oliver et al., 2000), and more specific details on syntheses and/or properties of specific compounds can be obtained from the corresponding author's website: www://barc.usda.gov/psi/caib/oliver.html

Bioassays were conducted using pods from an *Np/Np* pea line that had been selected from an F_4 heterozygote that was itself derived from a cross between C997-332 (*Np/Np*) and I_3 (*np/np*) (Doss et al., 1995). Plants were grown in a greenhouse with a minimum temperature of 16°C, and a maximum temperature of 21°C. Supplementary lighting was provided by high-pressure sodium lamps that were on from 06:00 until 22:00 hr, and a bank of eight fluorescent tubes (Sylvania F96T12/CW/SS) that were run continuously. Under these conditions, the development of spontaneously formed callus was kept to a minimum (Snoad and Matthews, 1969).

For bioassay, pods were removed from the plants at the late flat pod stage (Meicenheimer and Muehlbauer, 1982) and divided into halves by carefully splitting along the suture. The half pods, with immature seeds removed, were placed, with the outer surface up, upon moist filter paper in Petri dishes. Test solutions were applied as 1 μl drops in 50% (v/v) ethanol–water. Test compounds were applied at six levels: 1, 10, 100, 1,000, 10,000, and 100,000 fmol. Each compound was applied at each level to six separate pods using a random design. After test solutions on the pod were allowed to dry, the Petri dishes were covered and placed into a growth chamber (23°C, continuous fluorescent light, 30–40 $\mu E/m^2/sec$). Calli formed in response to application of test solutions were removed and weighed after one week.

RESULTS AND DISCUSSION

Synthesis. Analogs were synthesized by relatively straightforward procedures. The diol backbones of bruchin analogs were, in most cases, constructed by alkylation of appropriately substituted alkynes (see also Oliver et al., 2000), although other procedures included oxidative dimerization of ω-hydroxy-1-alkynes [Glaser coupling (Zweifel and Polston, 1970)] followed by hydrogenation. The diol portion of the symmetrical monoolefinic analog **10h** (Figure 2) was conveniently

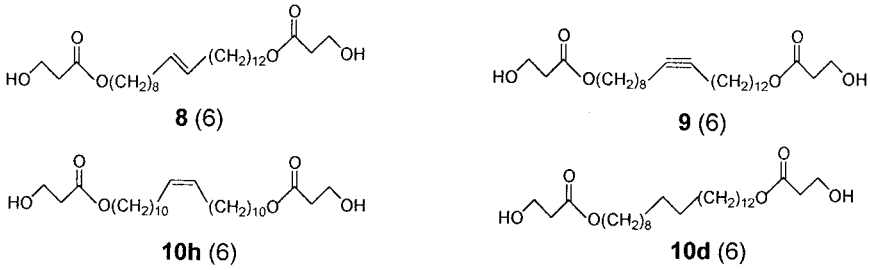


FIG. 2. Geometry, nature, and location of unsaturation (relative activity in parenthesis; see table).

prepared by oxidative dimerization of (11-hydroxyundecyl)triphenylphosphonium ylid (Poulain et al., 1996). The discovery that chain unsaturation was not a requirement for activity facilitated the evaluation of additional structural changes because synthetic procedures did not have to accommodate the unsaturation. Probably the most convenient and economical procedure for construction of 3-hydroxypropanoate esters consists of esterification of an alcohol or diol with 3-(benzyloxy)propanoyl chloride followed by catalytic hydrogenolysis (Oliver et al., 2000), a route not applicable in cases where unsaturation has to be maintained. That general route was used for the majority of the entries in Figures 3 and 4. For the comparison of mono versus diesters of α,ω-diols (Figure 3), it was often practical to react a given diol with slightly more than one equivalent of (3-(benzyloxy)propanoyl) chloride, then separate mono- and diesters by flash chromatography (mobility on silica gel decreased in the order diol > mono-(3-hydroxypropanoate) > bis-(3-hydroxypropanoate). In cases where catalytic hydrogenation was impractical (i.e., where chain unsaturation needed to be maintained), alcohols were esterified with 3-[(*t*-butyldiphenylsilyl)oxy]propanoyl

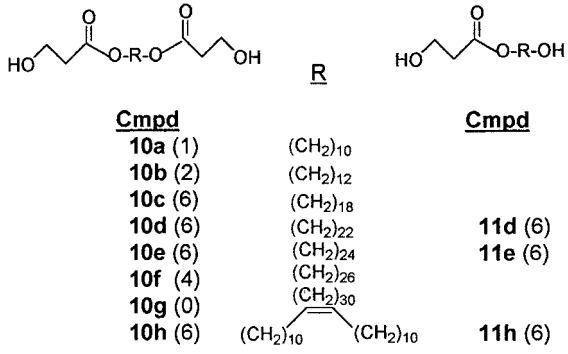
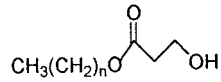


FIG. 3. Mono- and diesters of α,ω-diols (relative activity in parenthesis; see table).



n	Cmpd	n	Cmpd
0	**12a** (0)	23	**12e** (1)
9	**12b** (2)	25	**12f** (1)
21	**12d** (4)	29	**12g** (1)

FIG. 4. Chain length, monoesters of monoalcohols (relative activity in parenthesis; see table).

chloride, then the protecting group was removed with tetrabutylammonium fluoride (Oliver et al., 2000)). The *E* geometric isomer **8** was prepared by inverting the olefin geometry (Sonnet and Oliver, 1976) of the bis-(*t*-butyldiphenylsilyl) ether of **2** followed by deprotection. Diesters depicted in Figure 4 were made by standard techniques, using acid chlorides with protected functional groups in several cases, but simple Fisher esterifications in others. Analogs containing central aromatic rings (**13a–d**, Figure 5) were initiated by double Wittig condensations on phthalaldehydes or *p*, *p*′-biphenyldicarboxaldehyde followed by hydrogenation. The acrylate oxide **14f** was prepared from bisacrylate **14e** by bromoformylation followed by formate hydrolysis and base treatment (Ueno et al., 1974). For esters containing chiral centers, we normally chose racemic precursors to avoid stereochemical bias in the preliminary survey; an exception was **14d** where availability of L-serine was a deciding factor.

Dose–Response to Bruchin Application. Because the response of a pea pod to bruchin application is highly localized (Doss et al., 1995, 2000), and because only a limited amount of callus can form over a given area of the pod, it is possible to saturate the response rather easily. Accordingly, above a certain threshold, there may be little if any measurable effect of increased levels of application. For example, with naturally occurring compound **4**, activity was first observed with the 100 femtomole treatment, and there was no clear-cut effect of higher doses. Because of this, comparison of the relative activity of the various compounds was best made

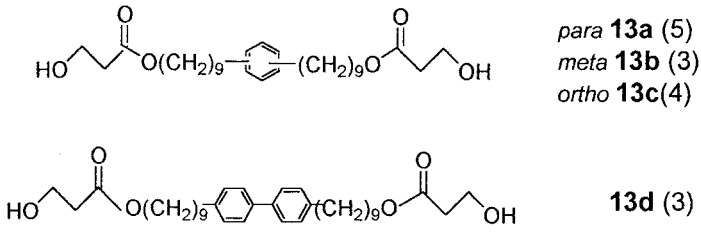


FIG. 5. Diesters of elaborated diols (relative activity in parentheses; see table).

by comparing the minimum active dose, which was taken to be the minimum dose with which half of the six treated pods developed measurable amounts of callus.

Table 1 reports mean weights (milligrams) of calli formed in response to application of test compounds. The column labeled "Score" provides a simple measure of the activity of the compounds where a score of 6 denotes a minimum active dose of 10^0 fmol; a score of 5, a minimum active dose of 10^1, etc. Hence, a score of zero indicates a compound that was inactive at all levels. The latter measure was useful for evaluating relative activities of the compounds.

Structural Parameters and Mitogenic Activity: Chain Unsaturation. All bruchins thus far identified from natural sources (**1**–**4**, Figure 1) contain a (*Z*)-9 double bond. Comparison of synthetic C_{22} analogs **8, 9, 10d,** and **10h** to natural product **2** (Figure 2) revealed that inversion [(*E*)-9-isomer **8**], dehydrogenation [9-alkyne **9**], relocation [(*Z*)-11 isomer **10h**] and removal altogether (saturated analog **10d**) of the double bond resulted in no measurable differences in activity, indicating that neither the presence nor location of unsaturation is particularly important. Moreover, the somewhat lesser activity of doubly unsaturated **4** indicates that increased unsaturation can be deleterious. We suspect that the (*Z*)-9 double bonds of **1**–**4** reflect lipid-based biosyntheses, although the issue has not been addressed experimentally.

Chain Length. After determining the apparent unimportance of unsaturation in the central chain, we synthesized a series of saturated bis- and mono-(3-hydroxypropanoates) with chain lengths ranging from 10 to 30 carbons (**10a**–**10g**, **11d–11h**, Figure 3). The most active compounds are mono- and bis-(3-hydroxypropanoates) of C_{18}–C_{24} α,ω-diols, e.g., **10c**–**10e**; these are the same chain lengths as the naturally occurring bruchins. Activity decreased markedly as chain length increased, and by C_{30} (**10g**) disappeared. It also decreased as the chain was shortened, although not quite as rapidly; barely detectable callus formation was observed at the highest applications of the C_{12} and C_{10} diesters (**10b** and **10a**, respectively.

Mono- versus Bis-(3-hydroxypropanoate) Esters of α,ω-Diols (*Figure 3*). Although Table 1 suggests monoester **1** to be slightly less active than corresponding diester **2**, the same comparison between several pairs of mono- and diesters of other diols, saturated and unsaturated, e.g., **11d–11h** versus **10d–10h** (Figure 3), indicated little or no difference in activity between the counterparts. In other words, removal of one 3-hydroxypropanoate from, for example, **2**, has little effect on activity, whereas removal of both eliminates it altogether. Thus two terminal OHs are decidedly advantageous, but only one 3-hydroxypropanoate ester is required.

3-Hydroxypropanoate Esters of Monoalcohols (Figure 4). Neoplasm formation was also observed following application of 3-hydroxypropanoates of long-chain primary alcohols (i.e., monoalcohols) **12b, 12c**–**12f** (Figure 4). However, the activities of the monoesters of monoalcohols were of a significantly lower level than those of mono esters of α,ω-diols of comparable chain length. Among

TABLE 1. CALLUS FORMED (mg) IN RESPONSE TO APPLIED COMPOUNDS[a]

Compound	Femtomoles applied						Score[b]
	10^0	10^1	10^2	10^3	10^4	10^5	
1[c]	0.6 ± 0.3	3.3 ± 0.3	2.0 ± 1.0	2.5 ± 0.5	2.3 ± 0.3	2.7 ± 0.6	5
2[c]	1.7 ± 0.5	1.5 ± 0.7	2.3 ± 0.5	2.8 ± 0.5	2.1 ± 0.6	3.2 ± 0.7	6
3[c]	1.3 ± 0.6	1.5 ± 0.5	2.3 ± 0.5	3.9 ± 0.7	2.1 ± 0.2	3.2 ± 0.6	6
4[c]	0.0 ± 0.0	0.0 ± 0.0	2.6 ± 0.4	2.6 ± 0.7	1.4 ± 0.3	3.1 ± 0.6	4
5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.5	0
8	2.0 ± 0.4	2.6 ± 0.6	3.1 ± 0.6	2.5 ± 0.5	1.4 ± 0.6	3.8 ± 0.7	6
9	2.5 ± 0.5	1.5 ± 0.9	2.4 ± 0.5	2.8 ± 0.5	1.5 ± 0.8	3.8 ± 1.0	6
10a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 1.6	1
10b	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.7	5.4 ± 0.9	2
10c	0.4 ± 0.2	2.0 ± 0.8	1.3 ± 0.3	3.9 ± 0.8	3.2 ± 0.7	4.4 ± 1.3	6
10d	0.9 ± 0.5	2.7 ± 1.5	2.2 ± 0.3	2.8 ± 0.2	2.1 ± 0.7	4.0 ± 0.5	6
10e	2.4 ± 0.9	4.5 ± 0.9	3.6 ± 1.2	3.9 ± 1.2	3.8 ± 1.1	6.4 ± 1.7	6
10f	0.0 ± 0.0	0.0 ± 0.0	3.0 ± 0.4	2.3 ± 0.6	2.6 ± 0.3	4.7 ± 1.6	4
10g	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
10h	2.7 ± 0.6	5.0 ± 0.1	2.9 ± 0.3	3.8 ± 0.7	2.1 ± 0.7	4.2 ± 1.1	6
11d	1.0 ± 05	2.3 ± 0.7	2.8 ± 0.7	3.2 ± 2.5	2.7 ± 0.6	3.3 ± 0.7	6
11e	2.4 ± 0.9	1.9 ± 0.9	3.1 ± 1.2	3.9 ± 1.2	3.8 ± 1.1	6.4 ± 1.7	6
11h	1.3 ± 0.9	1.9 ± 0.8	1.3 ± 0.6	2.0± 2.6	3.0 ± 0.4	3.2 ± 0.8	6
12a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
12b	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.5 ± 0.7	4.7 ± 0.7	2
12d	0.0 ± 0.0	0.0 ± 0.0	1.6 ± 0.4	6.1± 2.6	4.0 ± 0.7	3.8 ± 0.7	4
12e	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.9	4.5 ± 1.1	1
12f	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	1.2 ± 0.9	1
12g	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.9	4.5 ± 1.1	1
13a	0.2 ± 0.1	3.5 ± 0.8	3.6 ± 0.5	3.4 ± 0.7	3.6 ± 1.4	4.0 ± 0.8	5
13b	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.7 ± 0.3	1.2 ± 0.5	2.3 ± 0.5	3
13c	0.0 ± 0.0	0.0 ± 0.0	1.9 ± 0.5	3.1 ± 0.8	2.2 ± 0.8	3.6 ± 0.8	4
13d	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 0.6	1.8 ± 1.0	1.1 ± 0.7	3
14a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
14b	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
14c	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.2± 1.3	1
14d	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
14e	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
14f	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
14g	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
14h	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 0.3	3.3 ± 0.8	3.6 ± 0.7	3
14i	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
14j	0.0 ± 0.0	0.5 ± 0.5	3.8 ± 0.7	2.3 ± 0.6	3.4 ± 0.4	4.3 ± 0.7	4
14k	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.6 ± 0.6	2.9 ± 1.3	2
15	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
16	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
17	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
18	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
19	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0

[a] Callus formed (mg) in response to treatment of pea pods with synthetic compounds applied at six levels. Values are means ± standard errors for six blocks (pods).
[b] Score from 0–6, with 6 = most active, 0 = inactive (see text).
[c] Synthetic versions of natural bruchins isolated from pea weevil and cowpea weevil.

3-hydroxypropanoate esters of monoalcohols, the C_{22} analog **12d** was again the most active, and again activity decreased with increasing chain length until it had disappeared by C_{30} (**12g**). As had been the case with the difunctional counterparts, shortening the chain also led to decreased activity, although measurable activity was maintained through at least C_{10} (**12b**). The methyl ester of 3-hydroxypropanoic acid, (**12a**) was inactive, as was 3-hydroxypropanoic acid itself (**18**). To reiterate: although difunctionality is not an absolute necessity for activity, a terminal OH, even if not part of a (3-hydroxypropanoate) ester, decidedly contributes to optimum activity.

Bulkier and More Rigid Diol Backbones (Figure 5). In view of the apparently less stringent structural requirements for the central chain than for the acids constituting the ester termini, we synthesized some more elaborate analogs wherein aromatic rings were built into the centers of the molecules (**13a–d)**. One purpose was to address the reason for the importance of α, ω-difunctionality—how are two OHs at opposite ends of these long molecules involved in the same process? Having established that a chain length of about 22 carbons was optimum for activity, we tried to approximate that total length in some more rigid analogs. Models suggested that in contrast to the relatively flexible bruchins **1–4**, etc., the termini in analogs **13d** would be able to approach each other only with difficulty. Measurable activity was found for all of **13a–d**, with **13a** being the most active. Although the results do demonstrate the rather wide latitude of 3-hydroxypropanoate esters that can accommodate activity, the results may not contribute greatly to the question of flexibility and difunctionality, inasmuch as the activity of **13d** was no higher than that of some of the monofunctional 3-hydroxypropanoates, e.g., **12d** or **12e**.

The Acid Portion. A number of diesters of the saturated docosane-1,22-diol were synthesized and tested (Figure 6). In contrast to the diol chain, where within acceptable chain lengths some latitude exists for structural embellishment, it quickly became apparent that strict requirements are imposed on the ester termini. Some of the analogous esters could be considered as possible precursors or metabolites of 3-hydroxypropanoates, but few stimulated measurable cell division in the pea pod assay. Removal of the OH (propanoate ester **14a**), moving it one carbon in either direction (lactate **14b** or 4-hydroxybutanoate **14i**), or adding an additional heteroatom (serine or dihydroxypropanoate esters **14c** or **14d**) all greatly reduced or virtually eliminated activity. The corresponding diacrylate **14e** (a formal dehydration product) and its epoxide **14f** were equally inactive. Bis-(2,3-dihydroxypropanoate) ester **14c** exhibited marginal activity at the highest level of application, the elaborated ester **14j** (itself a 3-hydroxypropanoate) was modestly active, and 3-methoxypropanoate **14k** and the 3-hydroxybutanoate **14h** stimulated significant cell division in the pea pod assay. All of these were considerably less active than **10d**.

Hydrolysis Products (Figure 7). As mentioned, at least one 3-hydroxypropanoate is necessary for activity. Expected hydrolysis products [the α,ω-diols such as

Acyl-O(CH_2)$_{22}$O-Acyl

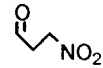
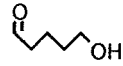
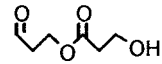
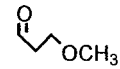
No	Acyl	No	Acyl
10d (6)		**14f** (0)	
14a (0)		**14g** (0)	
14b (0)		**14h** (3)	
14c (1)		**14i** (0)	
14d (0)		**14j** (4)	
14e (0)		**14k** (2)	

FIG. 6. Diesters of docosan-1,22-diol (relative activity in parentheses; see table).

5–7 (Figure 1) or **15–17**, (Figure 7)] were inactive. Similarly, 3-hydroxypropanoic acid itself (**18**) elicited no response in the pea pod assay. We have considered the possibility that intramolecular transesterification of a 3-hydroxypropanoate could generate propiolactone **19**, a known mutagen, but have failed in several attempts to trap **19** during hydrolysis of 3-hydroxypropanoate esters. Further, **19** stimulated no response in the pea pod assay.

In summary, we found that a long-chain (preferably about C_{22}, preferably difunctional) alcohol esterified with at least one 3-hydroxypropanoate moiety is essential for mitogenic activity. Moderate changes can be incorporated into the aliphatic portion of the molecules while retaining significant activity, whereas

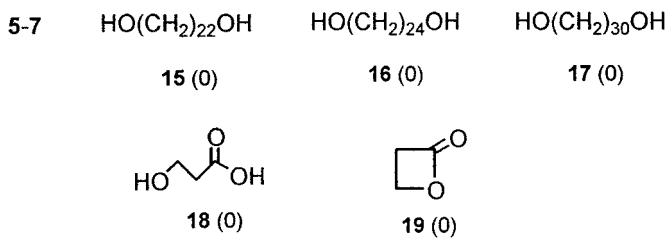


FIG. 7. Possible hydrolysis products (relative activity in parentheses; see table).

little alteration in the ester portion can be tolerated. The actual mechanism of mitogenic action remains under investigation. Transesterification involving 3-hydroxypropanoic acid remains a possibility, but logical hydrolysis products such as the α,ω-diols, 3-hydroxypropanoic acid, or propiolactone fail to promote cell division. Acyl transfer to an unknown cell component is certainly another possibility.

This study grew out of the unusual phenomenon of an insect-derived chemical stimulating neoplastic growth on pea pods (Berdnikov et al., 1992; Doss et al., 1995, 2000). Although callus formation in response to weevil oviposition appears to provide a measure of defense to the pea against entry of a pea weevil larva into the pea pod (Doss et al., 2000), the role or purpose of the bruchins in the insects remains unknown. While this manuscript was in preparation, we learned that Bartelt, Cossé, and coworkers (Bartelt, et al., 2002; Cossé et al., 2002) have identified acetates of unsaturated diols **5** and **6** as cuticular lipids of female wheat sawflies (*Cephus cinctus*) and have shown that these compounds serve as pheromone precursors via oxidative scission of the long chain. To the best of our knowledge, pheromone(s) have not been reported for *B. pisorum*, whereas pheromone components of *C. maculatus* have been reported to be several methyl-branched C_8-carboxylic acids (Phillips et al., 1996). The branched acids bear no obvious structural relationship to **1**–**4**, so at this time we have no reason to invoke a related function for these compounds.

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RIGHT STEREOISOMERS FOR SEX PHEROMONE COMPONENTS OF THE APPLE LEAFMINER, *Lyonetia prunifoliella*, IN KOREA

J. H. PARK,[1] K. S. HAN,[1] K. MORI,[2] and K. S. BOO[1,*]

[1]*School of Agricultural Biotechnology*
Seoul National University
Suwon 441-744, Korea

[2]*Insect Pheromone and Traps Division*
Fuji Flavor Co., Ltd.
Tokyo 205-8503, Japan

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Abstract—The sex pheromone of the American population of *Lyonetia prunifoliella* (Lepidoptera: Lyonetiidae) was reported to be composed of three components, 10,14-dimethyloctadec-1-ene (10me14me-1-ene-18Hy), 5,9-dimethyloctadecane (5me9me-18Hy), and 5,9-dimethylheptadecane (5me9me-17Hy). Studies with a Korean population showed that the 10*S*,14*S* isomer of the major compound was the major electrophysiologically by active stereoisomer. The 5*S*,9*S* isomers of the minar constituents also elicited electrophysiological responses. In field trappings, among the three components, the racemic mixture of 10me14me-1-ene-18Hy attracted *L. prunifoliella* males strongly, and the binary or tertiary combinations with racemic mixtures of the other two components did not increase trap catches. Among the four stereoisomers of 10me14me-1-ene-18Hy, only the 10*S*,14*S* isomer attracted *L. prunifoliella* males, as suggested by the EAG test, and attraction to the other three isomers was not observed. All stereoisomers of each minor component, as well as their racemic components, did not seem to have any additional effect on male attraction in Korea.

Key Words—Sex pheromone, *Lyonetia prunifoliella*, EAG, field trapping, 10,14-dimethyloctadec-1-ene, 5,9-dimethyloctadecane, 5,9-dimethylheptadecane.

INTRODUCTION

Lyonetia prunifoliella (Hübner) [formerly *L. speculella* (Schmitt et al., 1996)] (Lepidoptera: Lyonetiidea) is a small leafminer that damages apple leaves and

* To whom correspondence should be addressed. E-mail: ksboo@plaza.snu.ac.kr

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exists widely in Asia, America, and Europe. Females insert eggs singly into the lower surface of new leaves. Larvae form serpentine mines in the upper half of the leaves (Brown, 1989). Specifically, this moth damages new young trees and is increasing in apple orchards in Korea.

The sex pheromone of *L. prunifoliella* was identified for the North American population as composed of three components: the major one being 10,14-dimethyloctadec-1-ene (10me14me-1-ene-18Hy), and the two minor ones, 5,9-dimethyloctadecane (5me9me-18Hy) and 5,9-dimethylheptadecane (5me9me-17Hy) (Gries et al., 1997). Each of the three components has two chiral centers, so there are four stereoisomers for each chemical.

In Lyonetiidae, cases using specific stereoisomers as a sex pheromone have been reported (Sato et al., 1986; Tóth et al., 1989), but the pheromone chirality has not yet been investigated in *L. prunifoliella*, in part because of difficulty in synthesis and analysis. We synthesized all stereoisomers for the three components of the pheromone system in two different ways. Their activities were tested by EAG measurements and field trapping experiments for two years in Korea.

METHODS AND MATERIALS

Insects. Pupae of *L. prunifoliella* were collected in apple orchards in Gunwi, in the southern part of Korea, sexed, and kept at 22–25° and 60–70% relative humidity, under 16L:8D photoperiod. Adults were placed in Petri dishes containing moist filter paper coated with sugar. Females and males were kept separate to prevent mating.

Chemicals. The synthetic sex pheromone components were 10,14-dimethyloctadec-1-ene (10me14me-1-ene-18Hy), 5,9-dimethyloctadecane (5me 9me-18Hy), and 5,9-dimethylheptadecane (5me9me-17Hy). Their racemic mixtures were synthesized by G. Gries, as reported previously by Francke et al. (1988), Riba et al. (1990), and Gries et al. (1997). All the pure stereoisomers of the three chemicals were synthesized as reported by Tamagawa et al. (1999). The second set of the pure stereoisomer samples was synthesized according to Nakamura and Mori (2000).

Antennal Preparations for EAG Tests. For electroantennogram recordings, 3- to 7-day-old adult moths were momentarily anaesthetized with carbon dioxide. Their antennae, with both ends cut off, were mounted between the reference silver electrode and the recording silver electrode with electrode gel (Parker Laboratories Inc.).

EAG Tests. A charcoal-filtered airstream was blown continuously over the antennae via a stainless steel tube during every EAG test. Test compounds were dissolved in hexane, applied onto the filter paper and placed in a Pasteur pipet after evaporation of the solvent. A filter paper had 25–50 ng of each

component. The Pasteur pipet connected to the stimulus air controller (model CS-05) (Syntech, the Netherlands) was used for stimulation. Signals were amplified with a (UN-06) amplifier (Syntech), and displayed on the computer with interface. EAG tests were carried out with three or four insects for each isomer or racemic mixture. EAG response to chemicals was compared with that of the hexane (control).

Field Experiments. Field trappings were carried out in apple orchards in Gunwi, Korea, for two years, in 1999 and 2000. Rubber septum (IPM Technology Inc.), as a pheromone dispenser, was impregnated with a racemic mixture, a pure optical isomer of pheromone components, or their binary or tertiary mixtures in HPLC-grade hexane. The trap was baited with a rubber septum containing the chemicals. Butylated hydroxytoluene as an antioxidant was added to the lure in an amount equal to that of the pheromone components. Wing traps (IPM Technology Inc.) were hung on trees 1.5 or 2 m apart from each other. Traps were set up randomly irrespective of replicates ($N = 3$–5). Pheromone components were tested for their male attractivity in single, binary, or tertiary combinations with the racemic mixtures, or with pure stereoisomers for the three components of 10me14me-1-ene-18Hy, 5me9me-18Hy, and 5me9me-17Hy, which were referred to as compounds A, B, and C, respectively.

Statistical Analysis. Data from the EAG tests and field experiments were statistically analyzed by analysis of variance (ANOVA) with the SAS program (SAS Institute Inc., Cary, North Carolina, USA, 1996). If treatment means were significantly different at the 5% level or lower, they were compared by the least significance difference (LSD) test.

RESULTS

Electroantennograms. Among the four stereoisomers of the major component (10me14me-1-ene-18Hy), only the 10*S*,14*S* isomer elicited a strong electrophysiological response from the *L. prunifoliella* male antennae (Figure 1a). Other stereoisomers (10*S*,14*R*, 10*R*,14*R*, 10*R*,14*S*) were EAG-inactive. In the case of the two minor components (5me9me-18Hy and 5me9me-17Hy), the 5*S*,9*S* isomers also elicited an electrophysiologically strong response (Figures 1b,c), with the other stereoisomers (5*S*,9*R*, 5*R*,9*R*, 5*R*,9*S*) being EAG-inactive.

In binary combination tests, the EAG response to the 10*S*,14*S* isomer of 10me14me-1-18Hy did not seem to be affected by addition of other stereoisomers (10*S*,14*R*, 10*R*,14*R*, 10*R*,14*S*) (Figure 2a). Again, the EAG response of the *L. prunifoliella* male antennae to binary mixtures of the 10*S*,14*S* isomer of the major component with the 5*S*,9*S* isomer of each minor component was no different from the response to the single 10*S*,14*S* isomer of the major component (Figure 2b). Females did not respond to any stereosiomers or racemic forms of the three pheromone components in the EAG test.

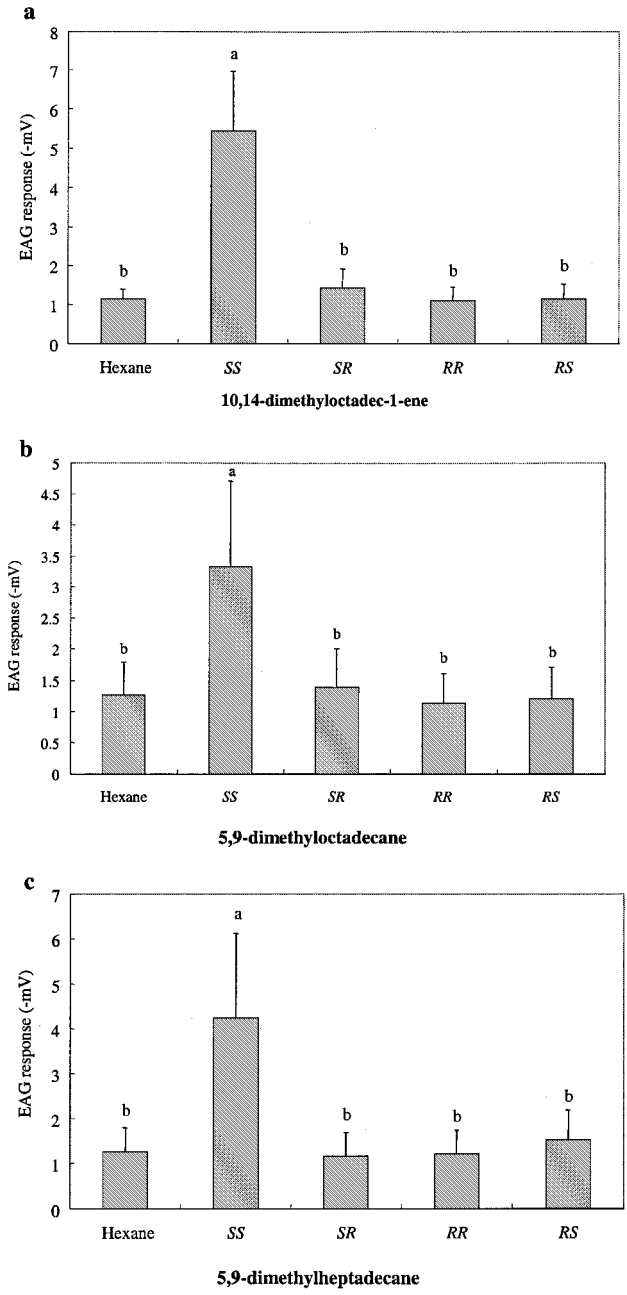


FIG. 1. The EAG response of *L. prunifoliella* male (virgin) antennae to four different stereoisomers of 10,14-dimethyloctadec-1-ene (a), 5,9-dimethyloctadecane (b), and 5,9-dimethylheptadecane (c) (50 ng each) ($N = 4$). Error bar indicates standard error.

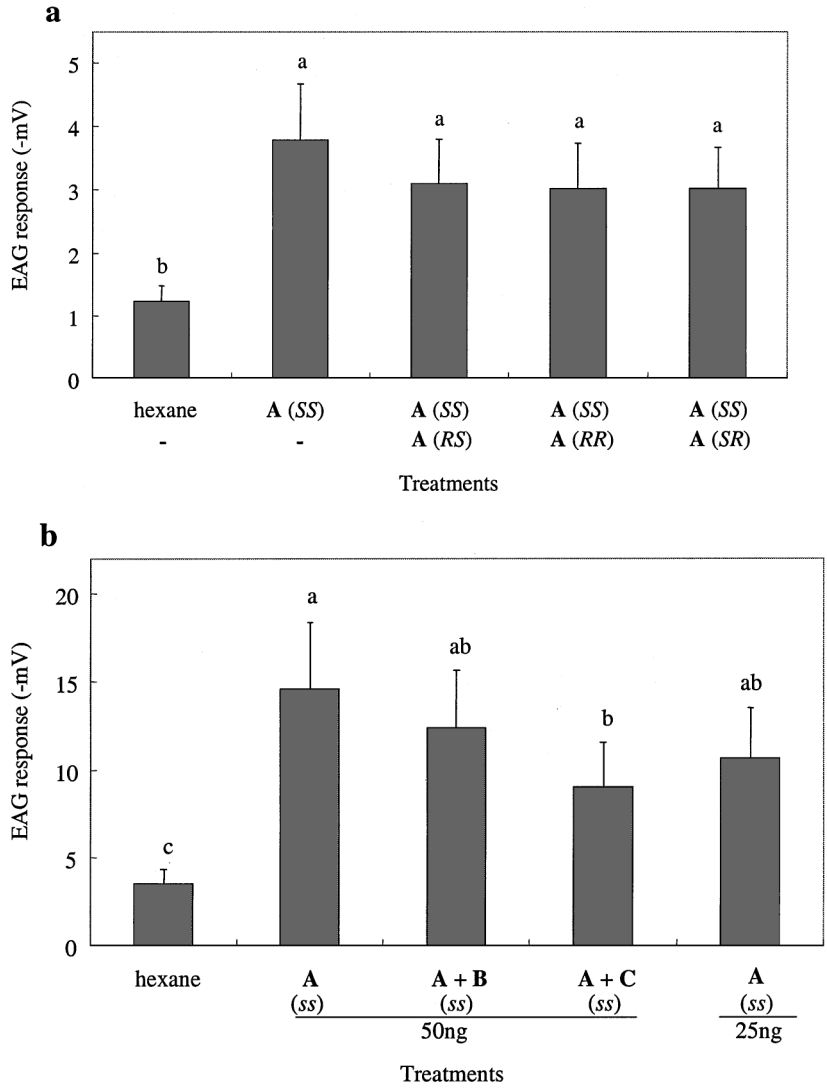


FIG. 2. The EAG response of *L. prunifoliella* male (virgin) antennae to binary mixtures of the 10*S*,14*S* isomer [A (*SS*)] of 10,14-dimethyloctadec-1-ene (A) with its other isomers [A (*RS*), A (*RR*), A (*SR*)] (50 ng each) (a), and to a single 10*S*,14*S* isomer [A (*SS*)] or its binary mixtures with each *SS* isomer of the other two minor components (b). (A: 10,14-dimethyloctadec-1-ene, B: 5,9-dimethyloctadecane, and C: 5,9-dimethylheptadecane) ($N = 4$). Error bar indicates standard error.

Field Experiments. By itself, the racemic form of the major component, 10me14me-1-ene-18Hy, attracted many *L. prunifoliella* males in the field (Figure 3a,b). Traps baited with its binary or tertiary combinations with 5me9me-18Hy and/or 5me9me-17Hy did not capture more males than those baited with the major component alone. A single treatment or a binary mixture of the racemic form of the two other minor components, 5me9me-18Hy and 5me9me-17Hy, did not attract *L. prunifoliella* males at all (Figure 3b).

Among the four stereoisomers of 10me14me-1-ene-18Hy, only the 10*S*,14*S* isomer attracted male *L. prunifoliella* (Figure 4). Catches in traps baited with 10*S*,14*R*, 10*R*,14*R*, or 10*R*,14*S* were not observed. In addition the 10*S*,14*S* isomer of 10me14me-1-ene-18Hy attracted many more *L. prunifoliella* males than an equal amount of its racemic mixture (Figure 4).

In tests with tertiary combinations of the 10*S*,14*S* isomer of 10me14me-1-ene-18Hy with eight stereoisomers and racemic forms of the two other minor components, only the 5*S*,9*S* isomer of 5me9me-18Hy slightly increased the number of males caught (Figures 5a,b).

DISCUSSION

This study demonstrates through EAG tests and field trapping experiments that among the three components reported to be sex pheromones for the North American population (Gries et al., 1997) of *L. prunifoliella*, the major component 10me14me-1-ene-18Hy alone is essential in attracting *L. prunifoliella* males in Korea. Among the four stereoisomers of the major component, the 10*S*,14*S* isomer alone showed activity in attracting *L. prunifoliella* males in the field, and no electrophysiological or field attractiveness of other stereoisomers was observed. This result is similar to that of *Leucoptera scitella* (Tóth et al., 1989) and *Lambdina fiscellaria* (Li et al., 1993), which have only one stereoisomer as the active pheromone component. In other species, more than one stereoisomer often affects attraction. For example, for *L. clerkella* using 14-methyl-1-octadecene as pheromone, the *S* isomer is the major component of the sex pheromone, and the addition of the *R* isomer to the *S* isomer had a synergistic effect (Sato et al., 1986). *Nepytia freemani* uses (3*S*,13*R*)-3,13-dimethylheptadecane as the major pheromone component, and the addition of any other isomer to the active *SR* isomer also increased attractiveness of the single 10*S*,13*R* isomer (King et al., 1995).

In the Korean population of *L. prunifoliella*, GC-EAD analysis confirmed that 10me14me-1-ene-18Hy was the major component (unpublished data), but the other two components could not be identified. In field tests, components attracting male moths in the Korean population seemed to be different from those in the American population. For the American population, *L. prunifoliella* males were attracted best to the tertiary mixture of the three components,

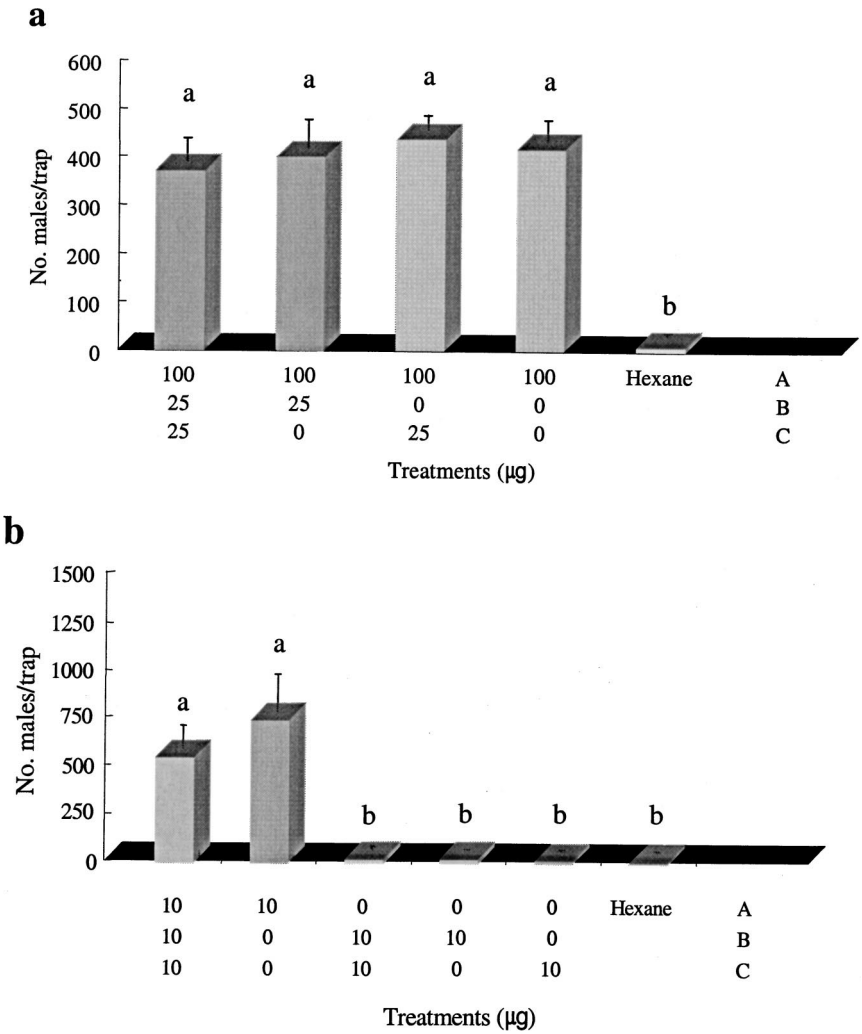


FIG. 3. The mean number of *L. prunifoliella* males caught in traps baited with a racemic mixture of 10,14-dimethyloctadec-1-ene (A), or its binary or tertiary mixture with the racemic form of the other two minor components in Gunwi (June 21–August 2, 1999) (a), and single or various mixtures of the racemic form of the three components in Gunwi (August 18–September 7, 1999) (b). (A: 10,14-dimethyloctadec-1-ene, B: 5,9-dimethyloctadecane, C: 5,9-dimethylheptadecane) ($N = 5$). Error bar indicates standard error.

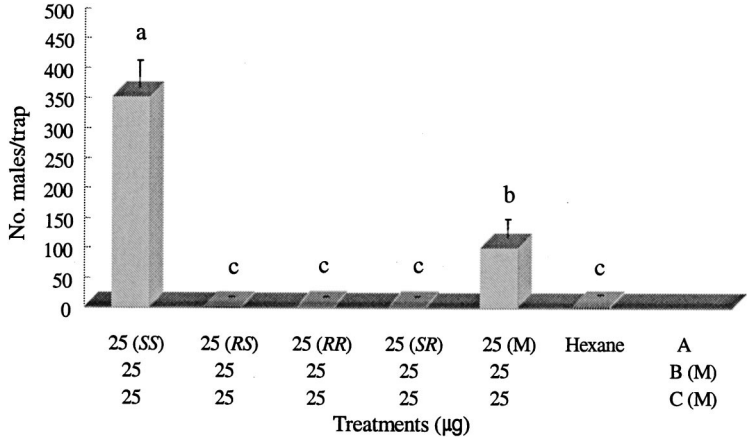


FIG. 4. The mean number of *L. prunifoliella* males caught in traps baited with tertiary mixtures of each stereoisomer or the racemic form (M) of 10,14-dimethyloctadec-1-ene (A) with the racemic form of the other two minor components 5,9-dimethyloctadecane (B) and 5,9-dimethylheptadecane (C), in Gunwi (June 2–21, 1999). ($N = 5$). Error bar indicates standard error.

10me14me-1-ene-18Hy, 5me9me-18Hy, and 5me9me-17Hy (Gries et al., 1997). Binary mixtures were also better than the major component alone. In contrast, in Korea, the major component, 10me14me-1-ene-18Hy, as a single component attracted *L. prunifoliella* males (Figures 3a,b). Binary or tertiary combinations with the other two components did not increase captures, even though *L. prunifoliella* males could electrophysiologically sense each *SS* isomer of the two minor components. Trap catches were slightly enhanced with the 5S,9S isomer of 5me9me-18Hy in tertiary combination with the other two components (Figure 5a). Racemic minor components did not affect attraction in any combination with the 10me14me-1-ene-18Hy (Figures 3a,b). The two minor components, especially the 5*S*,9*S* isomers, may have some other effect on mating, because the male moth could sense them. More research is needed to determine their function.

The reason for the difference between the American and Korean populations is not clear yet. One possibility is a difference among the leafminer species, perhaps at a subspecies level. The leafminer in Japan was reported to be *L. prunifoliella malinella* (Matsumura) (Yamada et al., 1970; Sekita and Yamada, 1979), different from those in America. Because Korea is close to Japan, the leafminer in Korea may be the same as that in Japan. However, the Korean leafminer has not yet been examined at the subspecies level. Such geographic variations in sex pheromone composition exist with many other insects and also with moths in Korea (Sugie et al., 1991; Cork et al., 1992). As an example, the optimum ratio for attracting

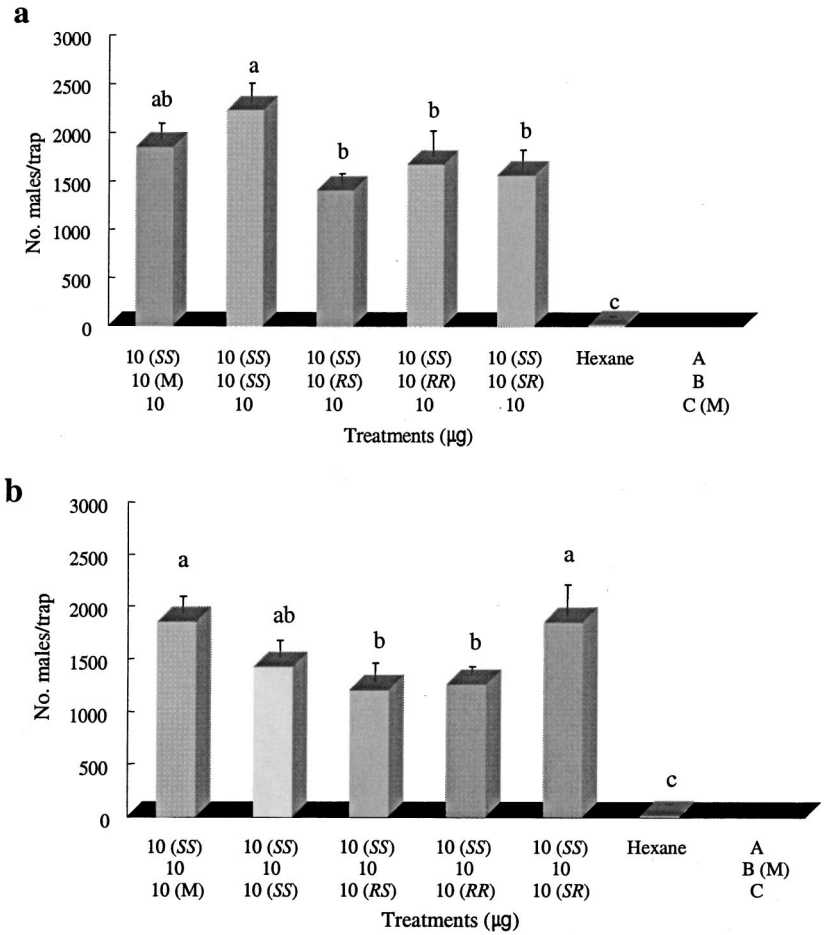


FIG. 5. The mean number of *L. prunifoliella* males caught in traps baited with tertiary mixtures of the 10*S*, 14*S* isomer of 10,14-dimethyloctadec-1-ene [A(*SS*)] with pure isomers or the racemic form of 5,9-dimethyloctadecane (B) and the racemic form of 5,9-dimethylheptadecane (C) (a), and with the racemic form of 5,9-dimethyloctadecane (B) and pure isomers or the racemic form of 5,9-dimethylheptadecane (C) (b) in Gunwi (August 18–September 7, 1999). ($N = 5$). Error bar indicates standard error.

Phyllonorycter ringoniella males in Korea [4:6 ratio of (*Z*)-10-tetradecenyl acetate and (*E*,*Z*)-4,10-tetradecadienyl acetate] was different from those reported in Japan (10:3) or China (7:3 to 6:4) (Boo, 1998). This trend of different pheromone compositions was more apparent among insect species with low mobility, at least in Korean cases (unpublished data).

Because the leafminer population is currently increasing in Korea, monitoring it in apple orchards is becoming important for its management. Since the two minor components, 5me9me-18Hy and 5me9me-17Hy, have no practical effect on field attraction of *L. prunifoliella* males, the major component, 10me14me-1-ene-18Hy, could be used by itself to monitor them in Korea. Moreover, the attractivity of the racemic mixture of the major component is sufficient when compared with that of two virgin females in the field (unpublished data). It would be more reasonable to use the racemic mixture because the synthesis of the pure isomer of 10*S*,14*S*-10me14me-1-ene-18Hy will be more expensive and time-consuming, and because other stereoisomers (10*S*,14*R*, 10*R*,14*R*, 10*R*,14*S*) in combination with the 10*S*,14*S* isomer do not have any negative or positive effect on male trapping.

Acknowledgments—This work was partly supported by the Brain Korea21 Project and a Korean Agricultural R & D Promotion Center project. We thank Prof. G. Gries of Simon Fraser University, Canada, for the generous gift of racemic mixtures of three pheromone components, and two anonymous reviewers for their valuable suggestions and comments in improving the original manuscript.

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SEMIOCHEMICALS OF THE SCARABAEINAE. VII: IDENTIFICATION AND SYNTHESIS OF EAD-ACTIVE CONSTITUENTS OF ABDOMINAL SEX ATTRACTING SECRETION OF THE MALE DUNG BEETLE, *Kheper subaeneus*

B. V. BURGER,* W. G. B. PETERSEN, W. G. WEBER, and Z. M. MUNRO

Laboratory for Ecological Chemistry
Department of Chemistry
University of Stellenbosch
Stellenbosch 7600, South Africa

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Abstract—Using gas chromatography with flame ionization detection (FID) and electroantennographic detection (EAD) in parallel, butanoic acid, skatole, and (*E*)-2,6-dimethyl-6-octen-2-ol were identified as constituents of the abdominal sex-attracting secretion of the male dung beetle, *Kheper subaeneus*, which reproducibly elicited EAD responses in male and female antennae. This is the first report of the occurrence of (*E*)-2,6-dimethyl-6-octen-2-ol as a natural product, for which the name (*E*)-subaeneol is proposed. In some experiments, a few other constituents of the secretion also gave reproducible responses in specific male and female antennae but did not elicit responses when the analyses were repeated with other antennae. The major volatile constituent of the secretion, identified as (*S*)-(+)-2,6-dimethyl-5-heptenoic acid, is one of these EAD-active compounds. Both this compound and (*E*)-2,6-dimethyl-6-octen-2-ol were synthesized from authentic starting materials for comparison with the natural products.

Key Words—Scarabaeinae, *Kheper subaeneus*, dung beetle, sex attractant, insect semiochemicals, electroantennography, terpenes, semiochemical synthesis.

INTRODUCTION

Male ball-rolling dung beetles of the genus *Kheper* produce a white, flocculant proteinaceous carrier material containing minute amounts of complex mixtures of volatile organic compounds acting as sex attractants for females. The secretion is extruded from several hundred tiny openings in a depression on either side of the

* To whom correspondence should be addressed. E-mail: lecus@sun.ac.za

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first abdominal sternite. It is then broken up into fine particles and released into the air by brushes on the tibia of the male. The ecological importance of dung beetles in general and biological aspects of the production and function of the abdominal sex attractant secreted by male *Kheper* dung beetles has been discussed in previous communications on this subject (Burger et al., 1983, 1990, 1995; Burger and Petersen, 1991, 2002). Hexadecanoic acid, 2,6-dimethyl-5-heptenoic acid, (*E*)-nerolidol, and skatole, were previously identified as the major volatile organic constituents of the abdominal secretion of *K. lamarcki.* (Burger et al., 1983). The abdominal secretion of *K. nigroaeneus* contains the EAD-active constituents (*R*)-3-methylheptanoic acid and skatole. A third EAD-active constituent of this secretion was not identified, as it is present in such a low concentration that it could barely be detected by gas chromatography (GC) using flame ionization detection (FID) (Burger and Petersen, 2002). A third *Kheper* species, *K. subaeneus*, occurs in parts of the Hluhluwe Game Reserve, about 50 km from the Mkuzi Game Reserve (both in South Africa) where *K. nigroaeneus* and *K. lamarcki* are found. In this communication, we report the identification of the EAD-active constituents of the abdominal sex-attracting secretion of male *K. subaeneus.*

METHODS AND MATERIALS

Collection of Material. We initially attempted to catch *K. subaeneus* dung beetles in pitfall traps baited with rhinoceros or horse dung in Hluhluwe Game Reserve, a method that was used effectively to catch *K. nigroaeneus* in Mkuzi Game Reserve. Probably due to the availability of large quantities of rhinoceros dung in the parts of the game reserve where these beetles are found, this method proved ineffective in catching *K. subaeneus* in Hluhluwe. *K. subaeneus* were, therefore, collected annually during the first week of November from 1988 to 1995 from rhinoceros dung middens along dirt roads near the main entrance gate of Hluhluwe Game Reserve. The dung beetles were transported to Stellenbosch, South Africa, and were kept in greenhouses. The secretion was collected as described in the previous paper in this series on semiochemicals of the Scarabaeinae (Burger and Petersen, 2002).

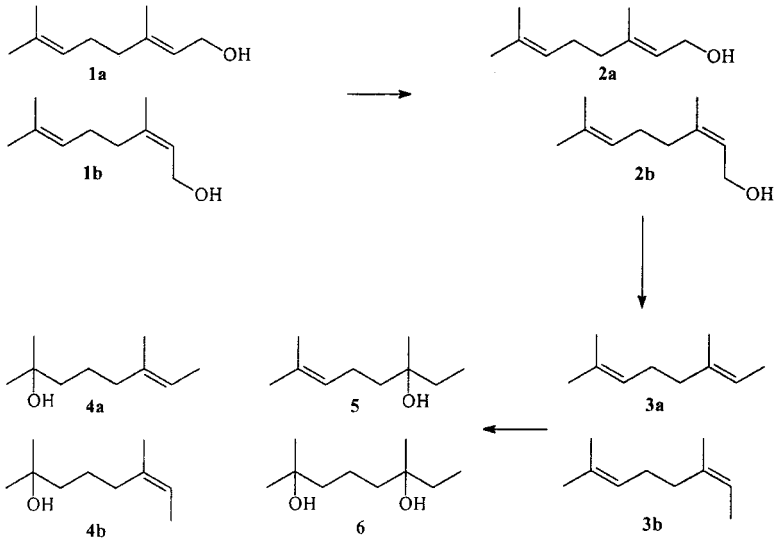
Analytical Methods. Gas chromatographic determinations were carried out with a Carlo Erba 4160 gas chromatograph with parallel flame ionization and electroantennographic detection (FID/EAD). The instrument was equipped with a 40-m $\times$ 0.3-mm glass capillary column coated with OV-1701-OH at a film thickness of 0.375 μm. Helium was used as carrier gas at a linear velocity of 28.5 cm/sec at 40°C, and a temperature program of 2°C/min from 40°C to 250°C (hold) was employed. Chiral gas chromatographic analyses were done on a glass column (25 m $\times$ 0.3 mm) coated with 10% heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin in OV-1701-OH at a film thickness of 0.25 μm, using H_2 as carrier gas at a linear velocity of 50 cm/sec. The column was programmed at 2°C/min from 40°C to

190°C (hold). The instrumentation for GC analysis of dung beetle secretions with FID and EAD recording in parallel using dung beetle antennae as sensing elements has been described by Burger and Petersen (1991). In short, an antenna was inserted lengthwise into the air duct, as this arrangement produced a less noisy baseline than positioning the antenna sideways in the airstream, a position in which the club of the antenna is subjected to the effect of the turbulent mixing of the effluent and ambient air. The antenna was protected against heat radiation from the gas chromatograph by a heat shield cooled with water at 18°C. Electrical responses in the antenna were recorded via Ag–AgCl electrodes placed in pipets filled with saline solution containing NaCl (7.5 g/liter), $CaCl_2$ (0.21 g/liter), KCl (0.35 g/liter), and $NaHCO_3$ (0.2 g/liter), as well as polyvinylpyrrolidone K90 (Fluka, 49 g/liter) to increase its viscosity (Van der Pers, 1980). Antennal responses were amplified with a Murphy Developments AMS-025 amplifier and were recorded on an Omniscribe recorder. Over a period of about 5 years, EAD/FID analyses were done with at least 80 male and as many female insects. Only one antenna of each dung beetle was used so that the beetle could still be used for the collection of secretion.

Mass spectra were recorded on a Carlo Erba QMD 1000 quadrupole mass spectrometer using the column and gas chromatographic conditions specified above. ^{1}H and ^{13}C NMR spectra of synthetic compounds and intermediates were recorded at 299.905 and 75.42 MHz, respectively, at 25°C on a Varian VXR 300 NMR spectrometer.

Reference Compounds. Commercially available reference compounds and starting compounds for the synthesis of other reference compounds were purchased from Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), or Sigma-Aldrich (Cape Town, South Africa).

(*E*)-2,6-Dimethyl-6-octen-2-ol (**4a**) was prepared from geraniol (3,7-dimethyl-2,6-octadien-1-ol) (**1a**) according to Scheme 1. Tetrachloromethane (69.3 g; 450 mmol) was added during 90 min to a stirred and chilled (0°C) solution of geraniol (**1a**) (61.6 g; 400 mmol) and triphenylphosphine (115.3 g; 440 mmol) in dry acetonitrile (350 ml). The temperature of the reaction mixture was allowed to rise gradually to 22°C at which temperature it was stirred for a further 19 hr. Pentane (300 ml) was added to the reaction mixture, which was stirred at room temperature for 15 min and left overnight at −15°C to allow precipitation of triphenylphosphine oxide. The precipitate was removed by filtration of the cold suspension. Pentane and acetonitrile were removed on a rotary evaporator, the remaining material diluted with pentane, the solution cooled to −15°C, and the precipitate filtered off. This procedure was repeated until no further triphenylphosphine oxide precipitated. Removal of the solvent and repeated fractionation gave a colorless oil (27.4 g; 39.7%), bp 106–107°C/22 mm Hg, containing (*E*)-8-chloro-2,6-dimethyl-2,6-octadiene (**2a**) in 97.6% purity (GC-MS). MS: m/z (%) = 159(0.3), 157(1), 136(10), 129(4), 121(9), 105(5), 93(57), 91(26), 79(25), 77(23), 69(100), 68(23), 67(23), 53(14), 41(53), 39(30). ^{13}C NMR ($CDCl_3$): $\delta = 41.17$ (t, C-1);



SCHEME 1.

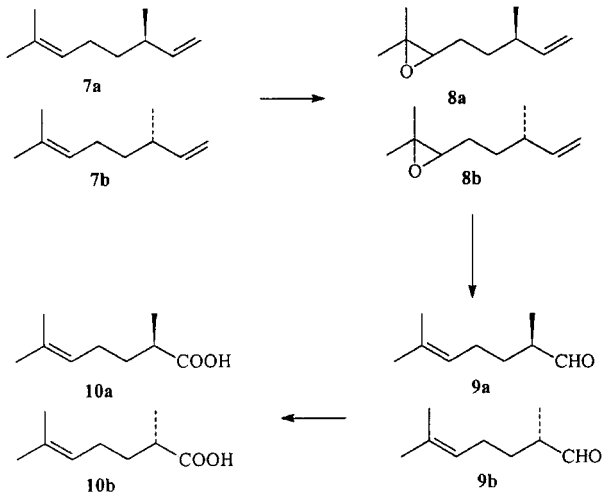
120.31 (d, C-2); 142.78 (s, C-3); 39.47 (t, C-4); 26.25 (t, C-5); 123.61 (d, C-6); 131.99 (s, C-7); 25.68 (q, C-8); 16.12 (q, CH_3C-3); 17.71 (q, CH_3C-7).

(*E*)-8-Chloro-2,6-dimethyl-2,6-octadiene (**2a**) (27.4 g; 159 mmol) was slowly added to a stirred solution of lithium aluminum hydride in tetrahydrofuran (THF) (380 ml; 0.02 g/ml; 200 mmol). The reaction mixture was refluxed for 4 hr, allowed to cool to room temperature, and diluted with pentane (150 ml). The precipitated inorganic material was filtered off. Conventional work-up procedures and distillation of the product gave a colorless oil (16.9 g; 77.2%); bp 77°C/ 40 mm Hg, containing 98.0% (GC-MS) of (*E*)-2,6-dimethyl-2,6-octadiene (**3a**); MS: m/z (%) = 138(3), 123(15), 109(1), 107(2), 95(38), 81(3), 79(3), 77(2), 69(100), 67(11), 55(5), 53(7), 41(53), 39(8). ^{13}C NMR ($CDCl_3$): δ = 26.38 (q, C-1); 131.98 (s, C-2); 125.11 (d, C-3); 27.45 (t, C-4); 40.42 (t, C-5); 136.49 (s, C-6); 118.93 (d, C-7); 14.02 (q, C-8); 18.34 (q, CH_3C-2); 16.34 (q, CH_3C-6).

Solid mercury(II) acetate (3.2 g; 10 mmol) was added in one batch to a stirred solution of the diene (**3a**) (1.38 g; 10 mmol) in THF (40 ml) and water (10 ml) at 22°C to produce a yellow reaction mixture (Brown et al., 1972). The reaction mixture became colorless after 6 min, and it was stirred for a further 2 hr. A solution of sodium hydroxide (1.2 g NaOH in 10 ml H_2O) was added in one batch to the reaction mixture, followed immediately by the rapid addition of a solution of sodium borohydride and sodium hydroxide (0.19 g $NaBH_4$ and 1.2 g NaOH in 10 ml H_2O). The temperature of the reaction mixture rose to 38°C, and mercury started to precipitate. The reaction mixture was stirred for 30 min,

after which the supernatant solution was decanted from the mercury and saturated with K_2CO_3, resulting in the separation of the THF layer containing the organic material from the saturated K_2CO_3 solution. Careful concentration of the THF phase on a rotary evaporator gave a mixture of the organic products (1.41 g) containing the starting material (**3a**), an unidentified product that could possibly be a cyclic ether formed from the starting material (Brown et al., 1972), the two terpenols (**4a**) and (**5**), and 2,6-dimethylocta-2,6-diol (**6**) in a ratio of 24:33:29:3:11. This procedure was repeated several times and the products (10.4 g) combined for column chromatographic fractionation on silica gel (450 g, Merck 60, 230–400 mesh). Elution with 10% ether in hexane gave a fraction (1.16 g) containing the desired terpenol (**4a**) and the terpenol (**5**) in a ratio of 97:3 (GC-MS). This fraction was subjected to bulb-to-bulb distillation (135°C/25 mm Hg) for spectrometric characterization. The major constituent was 2,6-dimethyl-6-octen-2-ol (**4a**); m/z (%) = (138)7, 123(20), 109(3), 95(6), 83(19), 82(100), 69(23), 67(80), 59(57), 55(23), 43(30), 41(3). ^{13}C NMR ($CDCl_3$): $\delta = 29.87$ (q, C-1); 71.67 (s, C-2); 44.17 (t, C-3); 23.31 (t, C-4); 40.67 (t, C-5); 136.31 (s, C-6); 119.15 (d, C-7); 13.95 (q, C-8); 29.87 (q, CH_3C-2); 16.14 (q, CH_3C-6). The minor constituent was 3,7-dimethyl-6-octen-3-ol (**5**); m/z (%) = 156(2), 141(17), 138(9), 127(8), 123(40), 113(13), 109(18), 95(13), 85(19), 81(18), 71(100), 69(25), 67(23), 58(19), 55(30), 53(12), 43(68), 41(45), 39(20).

(*R*)-(−)-2,6-Dimethyl-5-heptenoic acid (**10a**), was prepared from (*R*)-(−)-3,7-dimethyl-1,6-octadiene [(−)-β-citronellene] (**7a**) according to Scheme 2. A solution of 3-chloroperbenzoic acid (containing 6% 3-chlorobenzoic acid) (8.23 g;



SCHEME 2.

44.85 mmol) in chloroform (100 ml) was added over 1.25 hr to (*R*)-(−)-3,7-dimethyl-1,6-octadiene (**7a**) in chloroform (40 ml) at such a rate that the temperature of the reaction mixture did not exceed −10°C. The progress of the partial epoxidation of the β-citronellene was monitored by GC analysis and was completed after stirring the reaction mixture an additional 2 hr at −10°C. The 3-chlorobenzoic acid was filtered off, and the temperature of the filtrate allowed to rise to room temperature. The filtrate was washed with NaOH solution (0.45 M, 40 ml), Na_2CO_3 solution (5%), saturated brine and dried. Removal of the solvent under reduced pressure gave a colorless oil (6.87 g; 99.3%) containing 95.9% (GC-MS) of (R)-(−)-6,7-epoxy-3,7-dimethyl-1-octene (**8a**). MS: m/z (%) = 139(4), 125(2), 121(3), 111(6), 95(14), 85(13), 82(16), 81(100), 79(18), 71(30), 69(39), 68(50), 67(60), 59(64), 55(53), 54(40), 53(20), 43(44), 41(64), 39(37).

Ozone was passed through a solution of the epoxide (**8a**) (6.22 g, 40.32 mmol) in dry dichloromethane (350 ml) at −70°C until, after about 35 min, the reaction mixture developed a light purple color. The excess of ozone and oxygen was removed as quickly as possible by passing nitrogen through the reaction mixture for 40 min. This solution was carefully concentrated to 40 ml, cooled to 0°C, and added to a cold suspension of zinc (15.0 g; 229.4 mmol) in acetic acid (39 ml) containing sodium acetate (3.41 g; 41.52 mmol) and sodium iodide (10.72 g; 68.64 mmol) (Knöll and Tamm, 1975). The bulk of the dichloromethane was removed under reduced pressure, and the resulting solution was stirred for 2 hr at room temperature. The zinc was filtered off, and the solution of the reaction products in acetic acid was extracted repeatedly with dichloromethane. The combined extracts were washed with saturated brine and water, dried, and the solvent removed to yield a liquid product (5.52 g; 97.7%) containing 71.5% (GC-MS) of the target product, (*R*)-(−)-2,6-dimethyl-5-heptenal (**9a**). MS: m/z (%) = 140(3), 107(2), 97(1), 93(1), 91(2), 83(12), 82(100), 81(7), 70(5), 69(24), 67(46), 57(5), 56(16), 55(21), 53(10), 43(8), 42(7), 41(51), 39(16).

(*R*)-(−)-2,6-Dimethyl-5-heptenal (**9a**) was oxidized to the corresponding acid with chromic acid. Chromic acid solution (24 ml, containing 4.79 g of CrO_3 in 3 M H_2SO_4) was slowly added to a stirred solution of (*R*)-(−)-2,6-dimethyl-5-heptenal (**9a**) (3.85 g; 27.46 mmol) in acetone (40 ml). The color of the reaction mixture changed from orange to a dirty green, and a precipitate of inorganic salts was formed. The acetone solution of the organic products was decanted from the inorganic material, the acetone evaporated under reduced pressure, and the residual material dissolved in dichloromethane. The inorganic precipitate was dissolved in water (100 ml) and repeatedly extracted with dichloromethane. All of the dichloromethane extracts were combined and extracted with NaOH solution (3 M; 30 ml). This basic solution was extracted five times with dichloromethane to remove all of the unsaponified material, after which it was acidified with dilute sulfuric acid (3 M; 5.5 ml) and the organic acid extracted with dichloromethane. Conventional work-up methods and distillation

gave the target compound, (*R*)-(−)-2,6-dimethyl-5-heptenoic acid (**10a**) (2.07 g) in a yield of 48.3%, bp 112–114/5 mm Hg, $[\alpha]_D^{25} = -18.27^\circ$(neat), 95.7% ee (GC). MS: m/z (%) = 156(9), 138(13), 110(4), 95(7), 83(88), 82(49), 74(58), 69(46), 67(38), 55(48), 53(12), 41(100), 39(30). ^{13}C NMR ($CDCl_3$): δ = 183.04 (s, C-1); 38.84 (d, C-2); 33.56 (t, C-3); 25.62 (t, C-4); 123.48 (d, C-5); 132.42 (s, C-6); 25.68 (q, C-7); 16.86 (q, CH_3C-2); 17.65 (q, CH_3C-6).

(*S*)-(+)-2,6-Dimethyl-5-heptenoic acid (**10b**) was prepared from (+)-β-citronellene (**7b**) according to Scheme 2 and the procedures described for its *R* enantiomer. (*S*)-(+)-6,7-Epoxy-3,7-dimethyl-1-octene (**8b**) was obtained as a colorless oil (6.73 g; 97.3%) in a purity of 96.8% and converted to (*S*)-(+)-2,6-dimethyl-5-heptenal (**9b**), which was used without purification for the preparation of (*S*)-(+)-2,6-dimethyl-5-heptenoic acid (**10b**) (2.12 g; 48.1%). bp 117–118/5 mm Hg; $[\alpha]_D^{25} = +24.6^\circ$(neat), 96.3% ee (GC). MS: m/z (%) = 156(10), 138(14), 110(4), 95(7), 83(97), 82(52), 74(65), 69(48), 67(43), 55(50), 53(11), 41(100), 39(28). ^{13}C NMR ($CDCl_3$): δ = 183.02 (s, C-1); 38.82 (d, C-2); 33.55 (t, C-3); 25.61 (t, C-4); 123.45 (d, C-5); 132.44 (s, C-6); 25.68 (q, C-7); 16.86 (q, CH_3C-2); 17.65 (q, CH_3C-6).

RESULTS AND DISCUSSION

In their natural habitat, male *K. subaeneus* can often be observed exhibiting the typical secretion-producing behavior, which can be summarized as follows. Oriented with the head upwind and the front two pairs of legs on the dung surface, the male raises his hind legs and lowers his head into the dung. The legs are retracted simultaneously inwards towards the sides of the body and are then simultaneously extended. This movement results in the tibial brushes brushing the controlled-release proteinaceous material (Burger et al., 1990), extruded from the abdomen as fine fibers and containing the sex attractant, against the rows of curved abdominal bristles. This causes puffs of attractant to rise into the air from both sides of the insect. After a short time of approximately 20–30 sec, the retraction and extension of the hind legs are repeated. This procedure is kept up until a female arrives or the male is disturbed by other insects in or on the dung (Burger et al., 1983). Although their population density was rather low in Hluhluwe Game Reserve during the late 1980s, five or more males were often found calling on one large rhinoceros dung midden. In contrast, calling behavior has not been observed with the same frequency in *K. lamarcki* in Mkuzi Game Reserve. In captivity, however, this situation was reversed, and the secretion of male *K. subaeneus* was collected only in small quantities on only a few occasions. A total of only a few milligrams were collected over a period of 5 years from about 700 males. A gas chromatogram of the volatile organic fraction of the abdominal secretion of *K. subaeneus* is shown in Figure 1. The general appearance of this gas chromatogram is similar to that of

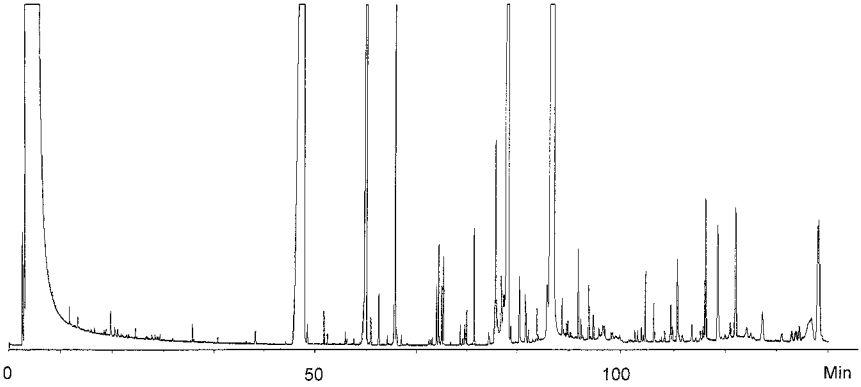


FIG. 1. Total ion chromatogram (GC-MS) of an extract of the male abdominal secretion of the dung beetle *Kheper subaeneus*. Gas chromatographic conditions are given in the text.

K. nigraeneus (Burger and Petersen, 2002) in that both secretions contain mostly heavy constituents. However, only a relatively small percentage of the compounds present are common to both secretions.

A typical GC analysis with FID and EAD recording in parallel using a female antenna as sensing element is shown in Figure 2. The constituent eluting at a retention time of 4.98 min was identified as butanoic acid (*n*-butyric acid) from its mass spectrum and coelution with the synthetic compound. Although the EAD response is weak, it was present in all EAD chromatograms. The constituent eluting at 28.27 min was identified as 3-methylindole (skatole) and is also present in the abdominal secretions of *K. lamarcki* (Burger et al., 1983) and *K. nigroaeneus* (Burger and Petersen, 2002). Although barely detectable by FID, the constituent eluting at 17.75 min gave a strong and reproducible EAD response. This constituent elutes with a shorter retention time than the EAD-active constituent that remained unidentified in the male abdominal secretion of *Kheper nigroaeneus* (Burger and Petersen, 2002). Male antennae produced similar EAD/FID analyses.

After the gas chromatographic analyses had been completed, only about ten times the quantity used for the analysis shown in Figure 2 was available for further analytical work. All of this material, collected during the previous five summer seasons, was used in a final attempt at obtaining some mass spectral information on the structure of the constituent eluting at 17.75 min. Using a very high electron multiplier setting, the mass spectrum shown in Figure 3 was obtained. The spectrum contains several ions typically found in the spectra of the monoterpenes. This and the presence of compounds with terpenoid character in the abdominal secretions of other *Kheper* species prompted us to compare the retention time and mass spectrum of the unknown compound with those of selected model terpenoid compounds. It became clear that the unknown compound was not a cyclic

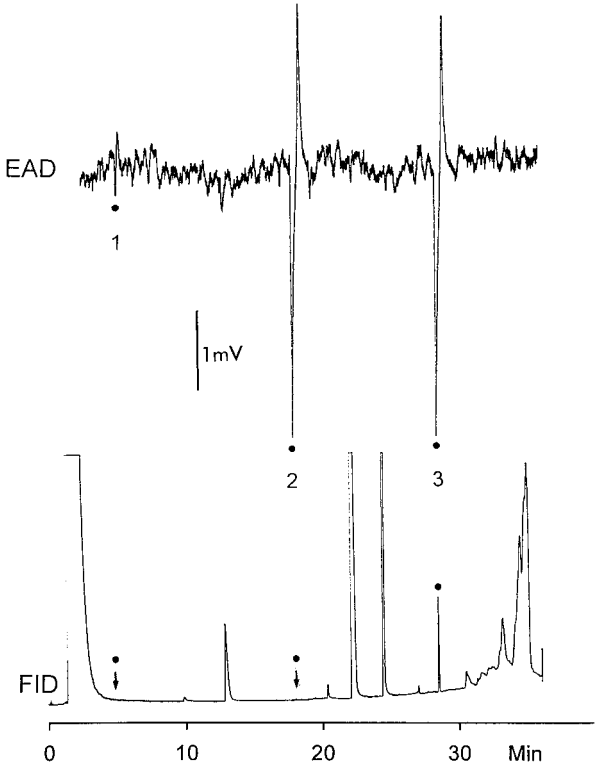


FIG. 2. First part of a gas chromatographic analysis of an extract of the male abdominal secretion of *Kheper subaeneus* with EAD and FID recording in parallel using a female antenna as sensing element. Chart speed was 0.5 cm/min. Gas chromatographic conditions are given in the text. The poor separation of later eluting peaks is due to overloading of the column. Assignment of EAD-active constituents: 1 = butanoic acid; 2 = (*E*)-2,6-dimethyl-6-octen-2-ol; 3 = skatole.

or acyclic monoterpene or a primary monoterpene alcohol and that it could probably be a tertiary monoterpene alcohol, albeit not one of the tertiary terpenoid alcohols that we had available for comparison. Partial hydroxylation of (*E*)- and (*Z*)-3,7-dimethyl-1,6-octadiene with mercury(II) acetate gave a mixture of alcohols containing, among others, 2,6-dimethyl-7-octen-2-ol, which had a retention time similar to that of the EAD-active constituent of the secretion but a slightly different mass spectrum. It was, therefore, concluded that the unknown constituent could possibly be a similar tertiary alcohol but with its double bond in a different position. Conversion of a mixture of geraniol (**1a**) and nerol (**1b**) [(*E*)- and (*Z*)-3,7-dimethyl-2,6-octadien-1-ol, respectively], via the corresponding chlorides (**2a**)

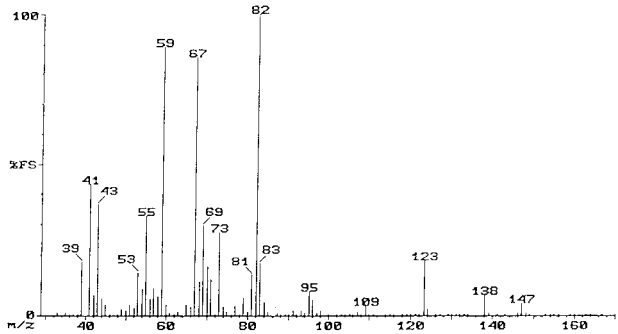


FIG. 3. Mass spectrum of the EAD-active constituent [(*E*)-2,6-dimethyl-6-octen-2-ol] of the male abdominal secretion of *Kheper subaeneus* eluting at 17.75 min in the gas chromatogram shown in Figure 2.

and (**2b**), to (*E*)- and (*Z*)-2,6-dimethyl-2,6-octadiene, (**3a**) and (**3b**), respectively (Scheme 1), and subjecting this mixture to partial hydroxylation, gave a mixture containing the two unchanged terpenes (**3a** and **3b**), the two terpenols (**4a** and **4b**), the terpenol (**5**), and the terpenediol (**6**). One of the terpenols was EAD-active and had the retention time and mass spectrum of the unknown compound. From the relative retention times of the three terpenols, it was concluded that the EAD-active one could possibly be (*E*)-2,6-dimethyl-6-octen-2-ol (**4a**).

The configuration of the double bond in the EAD-active compound was confirmed by repeating the synthesis with geraniol according to Scheme 1. Geraniol [(*E*)-3,7-dimethyl-2,6-dien-1-ol] (**1a**) was, thus, converted to (*E*)-8-chloro-2,6-dimethyl-2,6-octadiene (**2a**). Reduction of this chloride with lithium aluminum hydride gave the diene (**3a**). Partial hydroxylation of the diene (**3a**) by mercuration-demercuration according to Brown and Geoghegan, (1967) i.e., by the addition of the diene to a quantity of mercury(II) acetate equivalent to the hydroxylation of one of the double bonds, gave a mixture of the unchanged diene (**2a**), the target compound, (*E*)-2,6-dimethyl-6-octen-2-ol (**4a**)**,** 3,7-dimethyl-6-octen-3-ol (**5**), and 2,6-dimethyloctane-2,6-diol (**6**) in which the diols **4a** and **5** were present in an approximate ratio of 1:1. Although the two trisubstituted double bonds in the diene **3a** are so similar that totally regioselective hydroxylation was not expected to be feasible, reverse addition, i.e., addition of the mercury(II) acetate to the diene according to Brown et al. (1972), gave the terpenols **4a** and **5** in a ratio of 91:9. A fraction containing the desired terpenol, **4a**, and the terpenol **5** in a ratio of 97:3 was isolated from the crude product by column chromatography. As far as could be ascertained, (*E*)-2,6-dimethyl-6-octen-2-ol (**4a**) is a new natural product for which the name (*E*)-subaeneol is proposed.

In addition to butanoic acid, (*E*)-2,6-dimethyl-6-octen-2-ol, and skatole, which reproducibly elicited EAD responses in male and female antennae of *K.*

subaeneus, some other constituents of the abdominal secretion in a few instances also gave EAD responses. Because of the nonreproducibility of the EAD results, the minor EAD-active constituents of the secretion could not be identified. However, the major volatile constituent of the secretion, eluting at 47.2 and 22.1 min in the gas chromatograms shown in Figures 1 and 2, respectively, also gave an EAD response in about 5–10% of the analyses. This compound was identified as the chiral 2,6-dimethyl-5-heptenoic acid, which had previously been identified in *K. lamarcki* (Burger et al., 1983). (*R*)-(−)-2,6-Dimethyl-5-heptenoic acid (**9a**) was synthesized from commercially available (*R*)-(−)-3,7-dimethyl-1,6-octadiene [(−)-β-citronelene] (**7a**) by epoxidation of the trisubstituted double bond to give the unsaturated epoxide (**8a**), ozonolysis of the double bond in this compound, and simultaneous deprotection of the double bond (Knöll and Tamm, 1975) giving the unsaturated aldehyde (**9a**) and oxidation of the aldehyde to (*R*)-(−)-2,6-dimethyl-5-heptenoic acid (**10a**) according to Scheme 2.

Racemic 2,6-dimethyl-5-heptenoic acid could be resolved by gas chromatography on a capillary column coated with heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin/OV-1701. It was found that the synthetic acid (**10a**) and the natural compound did not coelute from this chiral column. Therefore, the natural compound had to be (*S*)-2,6-dimethyl-5-heptenoic acid (**10b**). This enantiomer, which coeluted with the natural compound was synthesized from (*S*)-(+)-3,7-dimethyl-1,6-octadiene [(+)-β-citronellene] (**7b**) according to a sequence of steps similar to those used for the synthesis of the *R* enantiomer (**10a**), as shown in Scheme 2.

Although the mass spectrum of the synthetic acid is identical to that of the natural compound and it coeluted with the natural compound from the chiral GC column, it did not elicit EAD responses in a large number of male and female antennae that were used in EAD/FID measurements. This is not unexpected in view of the observation that the natural compound also did not reproducibly elicit EAD responses. From the research done with dung beetles over many years, it is clear that semiochemical communication in dung beetles is extraordinarily complex, and as far as the inconsistency of antennal responses in EAD analyses with the antennae of this and other *Kheper* species is concerned, there are various factors that could be taken into consideration. It is, for example, possible that there could be some natural variation in the population in terms of antennal responses. Because the research had to be done with captured insects, it was not possible to determine the age, physiological state, etc of the insects that were used in these experiments, factors that could have influenced the EAD results. The possibility that the response of an antenna to the volatile constituents of the secretion could depend on the concentration of the components flowing over the antenna was investigated, but this does not seem to explain the inconsistent results obtained with (*S*)-2,6-dimethyl-5-heptenoic acid (**10b**) and some of the other minor constituents of the secretion. Another factor that has to be considered is that the best EAD results are mostly obtained when an EAD-active compound is eluted as a sharp peak

from the GC column. Better results are often obtained when higher programming rates are used. Many of the volatile constituents of the secretion are carboxylic acids or other polar compounds that elute as more or less pretailing peaks from apolar columns. If an EAD active constituent is eluted as a pretailing peak, it is possible that the receptors on the antenna become saturated at a level below the detection threshold of the system. In the present investigation, a capillary column coated with OV-1701-OH was used because it is known that underivatized fatty acids are eluted with better peak shapes from this phase than from other apolar and even moderately polar stationary phases. A detailed investigation of the effect of the choice of stationary phase and programming rate on EAD/FID analyses with dung beetle antennae have, however, not yet been undertaken. Using a modulating device, which delivers the effluent of the column as sharp pulses into the humidified airstream of the EAD setup, could possibly solve the problem of pretailing peaks in EAD analyses.

Unfortunately, as in the research on *K. nigroaeneus* (Burger and Petersen, 2002), field tests with synthetic materials have so far not produced positive results. The problems associated with field tests of the synthetic attractant constituents of *Kheper* dung beetles were discussed in a previous communication (Burger and Petersen, 2002).

The results obtained in interspecific EAD analyses revealed that considerable interspecific semiochemical communication could take place between dung beetles in the same area. This aspect will be dealt with in a forthcoming communication.

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IDENTIFICATION OF NEW HOMOTERPENE ESTERS FROM DUFOUR'S GLAND OF THE PONERINE ANT *Gnamptogenys striatula*

CLAUDIA M. SCHULZ,[1] LUTZ LEHMANN,[1] RUMSAÏS BLATRIX,[2] PIERRE JAISSON,[2] ABRAHAM HEFETZ,[3] and WITTKO FRANCKE[1,*]

[1]*Institut für Organische Chemie, Universität Hamburg*
Martin-Luther-King-Platz 6, D-20146 Hamburg, Germany

[2]*Laboratoire d'Ethologie Experimentale et Comparé (FRE CNRS 2473)*
Université Paris Nord
93430 Villetaneuse, France

[3]*Department of Zoology, George S. Wise Faculty of Life Sciences*
Tel Aviv University
Ramat Aviv, Tel Aviv 69978, Israel

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Abstract—Extracts of Dufour's gland of the ponerine ant, *Gnamptogenys striatula*, were analyzed by using the combination of gas chromatography and mass spectrometry. Series of esters of the new homoterpenoids (2*E*,6)-3,4,7-trimethyl-2,6-octadiene-1-ol (4-methylgeraniol) and (2*E*,6)-3,4,7-trimethyl-2,6-nonadiene-1-ol (bishomogeraniol) with unbranched medium-chain fatty acids were identified. Transformation of the chiral natural products into 1,4-di(trifluoroacetoxy)-3-methylpentane and comparison of its gas chromatographic retention time on a modified cyclodextrin phase with that of synthetic optically active reference samples proved the stereogenic center to keep (*S*)-configuration. (2*E*,4*S*,6)-3,4,7-Trimethyl-2,6-octadien-1-yl decanoate and the corresponding dodecanoate are the main volatiles in the extracts.

Key Words—*Gnamptogenys*, Dufour's gland, homoterpene, 4-methylgeraniol, terpenoid esters.

INTRODUCTION

Hymenoptera use various glands to produce chemical signals involved in intraspecific and interspecific systems of communication. In ants, Dufour's gland

* To whom correspondence should be addressed. E-mail: francke@chemie.uni-hamburg.de

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plays a complex role, and, depending on the genus and species, its secretion may contain a plethora of components. The chemical structures of the compounds may be highly diverse, ranging from straight-chain acetogenines and classical isoprenoides to methylated terpenes (Francke and Schulz, 1999).

Among the ponerine ants investigated so far, *Ectatomma ruidum* (Bestmann et al., 1995), *Gnamptogenys menadensis* (Gobin et al., 1998) as well as *G. striatula* (Blatrix et al., 2002) employ Dufour's gland secretion for trail marking. In this paper, we describe the structure elucidation of a few homoterpene esters as volatile components in Dufour's gland of *G. striatula*, representing a new class of natural products.

METHODS AND MATERIALS

Analyses. Pentane extracts of Dufour's glands of *G. striatula* were prepared as described by Blatrix et al. (2002). Analyses were carried out by coupled gas chromatography mass spectroscopy (GC-MS). A Hewlett-Packard gas chromatograph 5890 (Palo Alto, California, USA) was linked to a double-focusing sector-field mass spectrometer VG 70/250 SE (Vacuum Generators, Manchester, UK) operated at 70 eV. Alternatively, a quadrupole instrument (MD 800/GC 8060, Fisons, Ismaning, Germany) was employed. Using helium as carrier gas, separations were achieved with a 60 m $\times$ 0.25-mm-ID, 0.25-μm film DB5-MS fused silica column (J & W Scientific, Folsom, California, USA) under the following conditions: 2 min 50°C, then programmed to 280°C at a rate of 5°C/min. In addition, a 50-m $\times$ 0.25-mm-ID, 0.27-μm film FFAP fused silica column was operated under the following conditions: 3 min 50°C, then programmed to 220°C at a rate of 3°C/min. NMR spectra were recorded with a Bruker AMX 400 (Karlsruhe, Germany); chemical shifts (δ) are given in parts per million relative to tetramethyl silane; coupling constants J are given in Hertz.

The separation of stereoisomers of 1,4-di(trifluoroacetoxy)-3-methylpentane was carried out by using a 25-m $\times$ 0.25-mm-ID fused silica capillary coated with 6-methyl-2,3-dipentyl-γ-cyclodextrin operated at 55°C; hydrogen served as carrier gas.

Syntheses. Fine chemicals and solvents were purchased from Aldrich or Merck and were of highest purity available. Purifications of synthetic products were carried out by column chromatography on silica gel (Merck 60–240 mesh) using mixtures of hexane and ethyl acetate.

Preparation of racemic (2*E*,6)-3,4,7-trimethyl-2,6-octadiene-1-ol (4-methylgeraniol) and (2*E*,6)-3,4,7-trimethyl-2,6-nonadiene-1-ol (bishomogeraniol) followed the procedure of Ohnuma et al. (1989) (Figure 1). Starting from

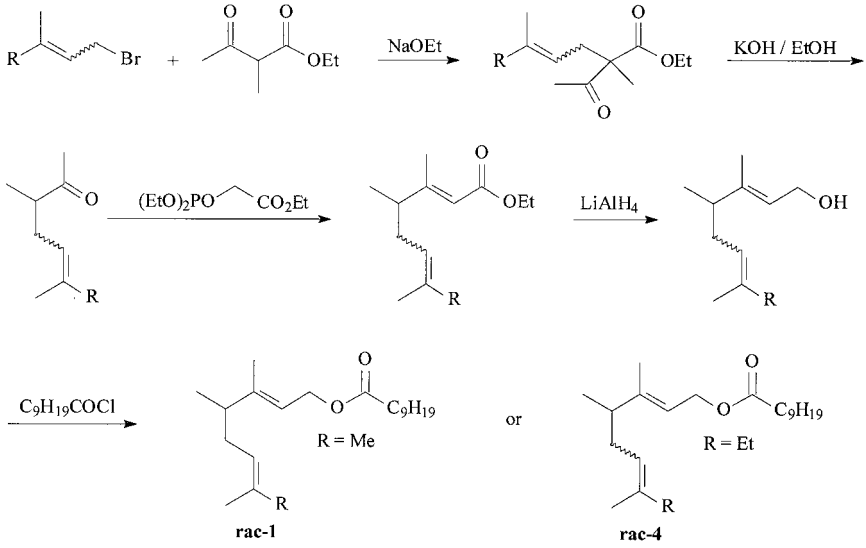


FIG. 1. Synthesis of homoterpene esters.

1-bromo-3-methyl-2-butene, the scope of the synthesis, work-up, and NMR data of 4-methylgeraniol were essentially the same as described in the literature . Similarly, our synthesis of racemic bishomogeraniol started with the reaction of 1-bromo-3-methyl-2-pentene with ethyl 2-methylacetoacetate. Terpenoid alcohols were esterified with fatty acid chlorides by standard procedures.

Spectroscopic data of (2*E*,6)-3,4,7-trimethyl-2,6-octadien-1-yl decanoate (4-methylgeranyl decanoate) (1) are as follows:

MS (70 eV): m/z [%] = 322 (0.04), 253 (4), 156 (8), 155 (75), 150 (15), 135 (18), 121 (7), 108 (9), 107 (39), 106 (6), 99 (18), 95 (27), 94 (15), 93 (6), 91 (5), 85 (22), 83 (9), 82 (49), 81 (30), 79 (11), 71 (33), 70 (6), 69 (93), 67 (50), 57 (41), 55 (37), 53 (16), 43 (62), 42 (12), 41 (100).

^{1}*H NMR (400 MHz, CDCl*$_3$*)*: δ [ppm] = 0.88 (t, 3H, J = 7.12 Hz, 10-H), 0.99 (d, 3H, J = 6.61 Hz, CH_3 linked to 4′-C), 1.21–1.34 (m, 12H, 4-H to 9-H), 1.55–1.69 (m, 11H, 3-H/8′-H/CH_3 linked to 3′-C), 1.98 (dd, 2H, J = 7.12 Hz, 5′-H), 2.08–2.16 (m, 1H, 4′-H), 2.29 (t, 3H, J = 7.12 Hz, 2-H), 4.59 (d, 2H, J = 7.12 Hz, 1′-H), 5.04 (t, 1H, J = 6.61 Hz, 6′-H), 5.33 (t, 1H, 2′-H).

^{13}*C NMR (101 MHz, CDCl*$_3$*)*: δ [ppm] = 13.62/14.11/17.85/18.82/25.77 (q, 10-C/8′-C/CH_3 linked to 3′-C/CH_3 linked to 4′-C), 22.67/24.37/25.05/29.17/29.27/29.43/31.88 (t, 3-C to 9-C), 33.49 (t, 5′-C), 34.44 (t, 2-C), 42.88 (d, 4′-C), 61.21 (1′-C), 117.72 (d, 2′-C), 122.92 (d, 6′-C), 132.09 (s, 7′-C), 146.12 (s, 3′-C), 173.94 (s, 1-C).

Spectroscopic data of (2*E*,6)-3,4,7-trimethyl-2,6-nonadien-1-yl decanoate (bishomogeranyl decanoate) (4) are as follows:

MS (70 eV): m/z [%] = 336 (0.02), 253 (4), 164 (12), 156 (8), 155 (71), 149 (10), 135 (19), 108 (9), 107 (28), 106 (5), 99 (11), 95 (22), 94 (12), 85 (20), 83 (62), 82 (79), 81 (25), 79 (12), 71 (35), 69 (14), 68 (5), 67 (64), 57 (24), 56 (7), 55 (100), 53 (11), 43 (26), 41 (39).

^{1}*H NMR (400 MHz, CDCl$_3$)*: δ [ppm] = 0.88 (t, 3H, J = 7.12 Hz, 10-H), 0.96 (t, 3H, J = 7.63 Hz, 9′-H), 0.99 (d, 3H, J = 6.61, CH_3 linked to 4′-C), 1.20–1.35 (m, 12H, 4-H bis 9-H), 1.55–1.68 (m, 8H, 3-H/CH_3 linked to 7′-C/CH_3 an 3′-C), 1.99 (q, 2H, 8′-H), 2.05 (dd, 2H, J = 6.61 Hz, 5′-H), 2.08–2.18 (m, 1H, 4′ H), 2.30 (t, 2H, J = 7.63 Hz, 2-H), 4.59 (d, 2H, J = 7.12 Hz, 1′-H), 5.04 (t, 1H, J = 7.63 Hz, 6′-H), 5.33 (t, 1 H, J = 6.61 Hz, 2′-H).

^{13}*C NMR (101 MHz, CDCl$_3$)*: δ [ppm] = 12.89/13.60 (q, CH_3 linked to 3′-C/CH_3 linked to 7′-C), 14.10 (q, 10-C), 18.80/18.86 (q, 9′-C/CH_3 linked to 4′-C), 22.68/24.84/25.05/29.17/29.27/29.44/31.88 (t, 3-C to 9-C), 32.46/33.28 (t, 5′-C/8′-C), 34.44 (t, 2-H), 42.93 (d, 4′-C), 61.20 (t, 1′-C), 117.72 (d, 2′-C), 121.40 (d, 6′-C), 133.62 (s, 7′-C), 146.99 (s, 3′-C), 173.54 (s, 1-C).

*Synthesis of 1,4-Di(Trifluoroacetoxy)-3-Methylpentane (**8**) (Figure 2A).* To a −78°C cold solution of 0.50 g (5.00 mmol) 3-methyltetrahydrofuran-2-one (**5**) in 8 ml hexane was added 6.5 mmol of diisobutylaluminum hydride (6.5 ml of a 1 M solution in heptane). The solution was slowly warmed to room temperature and added to 50 ml of a saturated aqueous solution of ammonium chloride. After stirring for 40 min, 20 ml of diethylether were added, and the organic layer was separated. The aqueous solution was extracted 3 times with diethylether, and the organic layers were combined, washed with brine, dried over anhydrous magnesium sulfate, and concentrated *in vacuo* to yield the lactol **6**. This was immediately converted to the diol **7:**. To a −78°C cold solution of 300 mg (2.88 mmol) crude **6** in 4 ml dry tetrahydrofuran was added 5 mmol methyl lithium (5 ml of a 1 M solution in hexane). After warming to room temperature, the solution was poured on ice/diethylether. After separation of the organic layer, the aqueous phase was extracted 8 times with portions of 20 ml of dichloromethane. The combined organic layers were washed with brine, dried over magnesium sulfate, and concentrated *in vacuo*. The crude 3-methyl-1,4-pentanediol (**7**) was purified through column chromatography. Trifluoroacetylation (Blau, 1993) of **7** yielded a mixture of all stereoisomers of 1,4-di(trifluoroacetoxy)-3-methylpentane (**8**).

^{1}*H NMR (400 MHz, C$_6$D$_6$)*: δ [ppm] = 0.49 (d, 3H, J = 6.87 Hz, CH_3 linked to 3-C), 0.98–1.03 (m, 3H, 1-H), 1.07–1.13 (m, 1H, 3-H), 1.59–1.65 (m, 2H, 4-H), 3.58–3.69 (m, 2H, 2-H), 3.79–4.00 (m, 2H, 5-H).

^{13}*C NMR (101 MHz, C$_6$D$_6$)*: δ [ppm] = 14.43/14.60 (q, CH_3 linked to 3-C), 25.11/26.96 (q, 1-C), 29.48/30.26 (t, 4-C), 40.68 (d, 3-C), 66.62 (d, 2-C), 67.55 (t, 5-C). (Trifluoracetylgroups not visible)

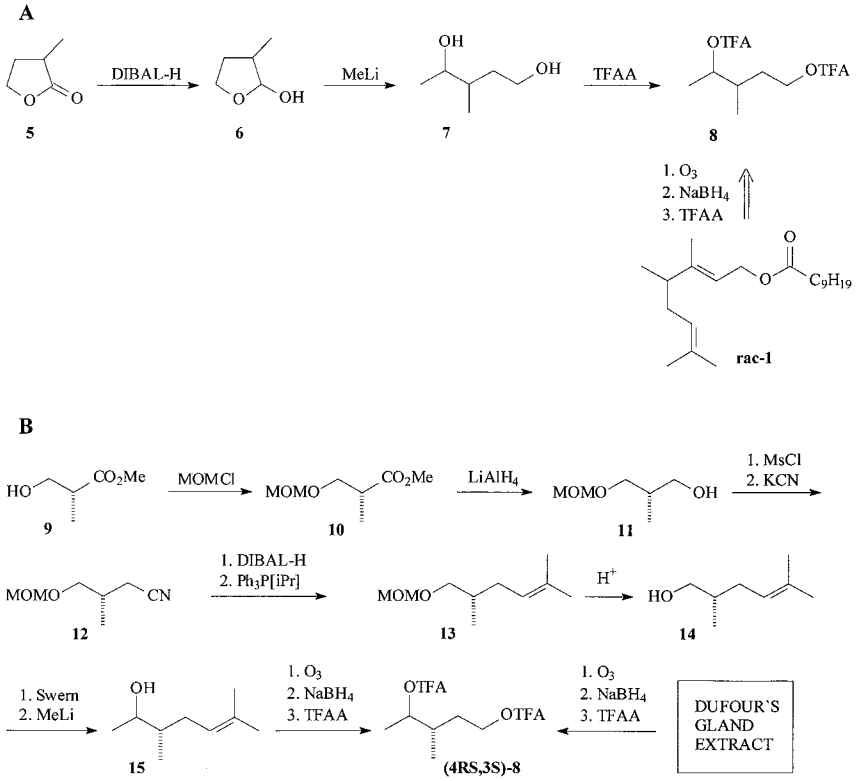


FIG. 2. (A) Synthesis of racemic 1,4-di(trifluoroacetoxy)-3-methylpentane and degradation of synthetic 4-methylgeranyl decanoate; (B) synthesis of (3*S*,4*RS*)-1,4-di(trifluoroacetoxy)-3-methylpentane and degradation of naturally occuring homoterpene esters.

MS: 268 (1), 228 (1), 210 (1), 181 (2), 169 (7), 142 (4), 141 (42), 113 (17), 99 (6), 97 (11), 84 (3), 83 (36), 82 (15), 70 (2), 69 (100), 67 (19), 56 (15), 55 (89), 53 (11), 47 (21), 45 (3), 43 (3), 41 (30), 39 (14).

*Synthesis of (3*S,*4*RS*)-1,4-Di(Trifluoroacetoxy)-3-Metehylpentane (3*S,*4*RS*)-**8** (Figure 2B). Methyl (*R*)-3-methoxymethoxy-2-methylpropanoate (**10**).* To an ice cold solution of 10.0 g (84.7 mmol) commercially available methyl (*R*)-3-hydroxy-2-methylpropanoate (**9**) and 21.8 g (169 mmol) diisopropyl amine in 50 ml dry dichloromethane, 10.2 g (127 mmol) chloromethylmethylether was added dropwise. After stirring for 4 hr at room temperature, the mixture was poured on ice. The organic layer was separated, and the aqueous layer was extracted 3 times with diethylether. The combined organic solutions were washed with a saturated aqueous solution of ammonium chloride and brine and dried over magnesium sulfate. After concentration *in vacuo*, column chromatography yielded 12.8 g (79 mmol, 93%)

of methyl (*R*)-3-methoxymethoxy-2-methylpropanoate (**10**) as a light yellow oil $[\alpha]_D = -12.5$ ($c = 2.31$ in $CDCl_3$)

^{1}*H NMR (400 MHz; CDCl$_3$)*: (δ [ppm]; *J* (Hz): 4.62 (2H, m, H-1), 3.36 (3H, s, MeO), 3.50–3.44 (1H, m, H-4), 3.39–3.34 (1H, m, H-4′b), 2.55 (1H, dd, H-2á), 2.22 (1H, dd, H-2′b), 2.31–2.24 (1H, m H-3′), 1.02 (3H, d, Me-3). $J_{2a,2b} = 5.3$, $J_{2a,3} = 15.3$, $J_{2b,3} = 7.6$, $J_{Me-2} = 6.6$.

^{13}*C NMR (101 MHz, CDCl$_3$, DEPT)*: δ [ppm]: 178.6 (C-1), 96.5 (C-1′), 72.0 (−), 55.2 (+), 38.3 (+), 30.6 (−), 16.9 (+).

MS: 162 (2), 147 (14), 131 (8), 103 (21), 69 (100), 45 (26).

*(S)-3-Methoxymethoxy-2-Methylpropanol (**11**).* To a −5°C cold suspension of 3.42 g (90 mmol) lithium aluminum hydride in 120 ml dry diethylether was added a solution of 12.6 g (78 mmol) of **10** in 20 ml dry diethylether. After stirring at room temperature over night, 50 ml diethylether were added. To the vigorously stirred mixture were added 14 ml of a 2 M aqueous solution of sodium hydroxide. After stirring for 1 hr, the precipitate was filtered off, and the residue was washed with diethylether. The combined organic layers were dried over magnesium sulfate, and the solvent was removed *in vacuo*. The obtained crude (*S*)-3-methoxymethoxy-2-methylpropanol (**11**), $[\alpha]_D = -5.68$ ($c = 2.50$ in CH_2Cl_2), was used for the next step without further purification.

^{1}*H NMR (400 MHz; CDCl$_3$)*: δ [ppm]; *J* (Hz): 3.64–3.58 (2 H, m, H-1), 3.52–3.45 (2H, m, H-3), 3.34 (3H, s, MeO), 3.63 (2H, s, OCH_2O), 2.08-1.98 (1H, m, H-2), 0.92 (3H, d, Me-2). $J_{2,Me} = 7.1$.

^{13}*C NMR (101 MHz, CDCl$_3$, DEPT)*: δ [ppm]: 96.6 (−), 72.1 (−), 67.1 (−), 55.3 (+), 35.6 (+), 13.5 (+).

*(S)-4-Methoxymethoxy-3-Methylbutyronitrile **12**.* Crude **11** obtained in the previous step (8.9g = 66 mmol) was taken up with a solution of 11.1 g (110 mmol) of triethylamine in 130 ml dichloromethane. After cooling to −10°C, 10.67 g (93.6 mmol) of mesylchloride were added. After stirring for 30 min, the solution was poured on ice. The organic layer was separated, and the aqueous layer was extracted 3 times with portions of 40 ml diethylether. The combined organic layers were washed with brine and dried over magnesium sulfate. Removal of the solvent *in vacuo* yielded (*S*)-1-mesyloxy-3-methoxymethoxy-2-methylpropane, which was immediately used for the next step: The mesylate, obtained in the previous step, was dissolved in 180 ml dry dimethylsulfoxide, and 7.61 g (117 mmol) potassium cyanide were added. The solution was stirred over night at 40°C and subsequently poured on ice. The aqueous mixture was saturated with sodium chloride and extracted 6 times with portions of 50 ml diethylether. The combined organic solutions were washed with brine and dried over magnesium sulfate. After removal of the solvent *in vacuo*, the product was purified by column chromatography yielding 8.69 g (60.8 mmol = 75 % starting from **10**) of pure (*S*)-4-methoxymethoxy-3-methylbutyronitrile, **12** $[\alpha]_D = -1.06$ ($c = 2.84$ in $CHCl_3$).

^{1}H NMR (400 MHz; $CDCl_3$): δ [ppm]; J(Hz): 4.62 (2H, s, H-1), 3.53 (1H, dd, H-3a′), 3.48 (1H, dd, H-3′b), 3.37 (1H, s, MeO), 2.50 (1H, dd, H-1a′), 2.39 (1H, dd, H-1b′), 2.22–2.11 (1H, m, H-2′), 1.10 (1H, d, Me-2).

^{13}C NMR (101 MHz, $CDCl_3$, DEPT): δ [ppm]: quarternary C not observed, 96.6 (−), 70.6(−), 55.4(+), 31.1(+), 21.4 (−), 16.3(+).

MS: 142 (4; M^+-1), 113 (8), 82 (18), 55 (22), 45 (100).

*(S)-1-Methoxymethoxy-2,5-Dimethylhex-4-ene (**13**).* To a −78°C cold solution of 8.65 g (60.5 mmol) of the nitrile **12** in 70 ml hexane 67 mmol of diisobutylaluminum hydride (67 ml of a 1 molar solution in heptane) was added dropwise. During a period of 3 hr the mixture was slowly warmed to −20°C. Subsequently, the mixture was warmed to 0°C and poured on to a mixture of 50 ml hexane and 80 ml of a saturated aqueous solution of ammonium chloride and vigorously stirred for 1 hr. The layers were separated, and the aqueous layer was extracted 4 times with portions of 50 ml diethylether. The combined organic layers were washed with brine and dried over magnesium sulfate. After removal of the solvent *in vacuo*, the residue was filtered over silica to yield 8.33 g (49.0 mmol = 81%) of the target aldehyde, (*S*)-4-methoxymethoxy-3-methylbutanal, which was immediately used in the next step: To a −30°C cold suspension of 25.9 g (60 mmol) isopropyl triphenylphosphonium iodide (purchased from Lancaster) in dry tetrahydrofuran was added 60 mmol *n*-butyl lithium (37.5 ml of a 1.6 M solution in hexane). The red solution was stirred for 20 min and cooled to −50°C. Subsequently, a solution of 7.5 g (50 mmol) of (*S*)-4-methoxymethoxy-3-methylbutanal in 20 ml tetrahydrofuran was added. The mixture was warmed to room temperature and added to an ice cold, vigorously stirred mixture of 80 ml water and 80 ml hexane. The layers were separated, and the aqueous phase was extracted 4 times with portions of 30 ml hexane. The combined organic solutions were washed with brine and dried over magnesium sulfate. The crude product was purified through column chromatography yielding 6.71 g (39 mmol = 78%) of (*S*)-1-methoxymethoxy-2,5-dimethylhex-4-ene, **13**, as a light yellow oil, $[\alpha]_D^{25} = +2{,}71$ ($c = 0.4425$ in hexane).

^{1}H NMR (400 MHz; $CDCl_3$): δ [ppm]; J(Hz): 5.16–5.10 (1H, m, H-4′), 4.62 (2H, s, H-1), 3.39 (1H, dd, H-1′a), 3.36 (3H, s, MeO), 3.30 (1H, dd, H-1′b), 2.08 (1H, m, H-3′a), 1.87 (1H, m, H-3′b), 1.70 (3H-, s, Me-5), 1.60 (3H, s, Me′-5′), 0.93 (3H, d, Me-2′). $J_{1'a,1'b} = 9.2$, $J_{1'a,2'} = 6.1$, $J_{1'b,2'} = 6.6$, (3H, d, Me-2).

^{13}C NMR (101 MHz, $CDCl_3$, DEPT): δ (ppm): 132.5 (q), 122.5 (C-4′), 96.6 (C-1), 72.9 (C-1′), 55.1 (MeO), 34.3 (+), 32.0 (−), 25.8 (+), 17.8 (+), 16.9 (+).

*(S)-2,5-Dimethylhex-4-en-1-ol (**14**).* To a stirred solution of 3,0 g (17,4 mmol) of **13** in 30 ml methanol were added 5 drops of concentrated hydrochloric acid at room temperature. After 5 hr the mixture was cooled to 0°C and neutralized by slowly adding solid sodium hydrogen carbonate. After removal of ca. 70% of the solvent *in vacuo*, 40 ml water and 40 ml diethylether were added. The layers were separated, and the organic layer was extracted 5 times with portions of 40 ml diethylether. The combined organic layers were washed with brine and dried over

magnesium sulfate. The crude product was purified by column chromatography yielding 2.00 g (15.7 mmol = 90.0%) of (*S*)-2,5-dimethylhex-4-en-1-ol, **14**, as a colorless oil $[\alpha]_D^{25} = -0.562$ ($c = 1.38$ $CHCl_3$).

^{1}*H NMR (400 MHz; CDCl$_3$)*: δ [ppm]; J(Hz): 3.52 (1H, dd, H-1a), 3.44 (1H, dd, H-1b), 2.11–2.04 (1H, m, H-3a), 1.92–1.84 (1H, m, H-3b), 1.73–1.66 (1H, m, H-2), 1.71 (3H, s, Me-5), 1.62 (3H, s, Me-5), 0.92 (3H, d, Me-2). $J_{1,1} = 10.7$, $J_{1,2} = 6.1$, $J_{2,\mathrm{Me}} = 6.6$.

^{13}*C NMR (101 MHz, CDCl$_3$, DEPT)*: δ [ppm]: 132.8 (q), 122.6 (C-4), 68.2 (C-1), 31.9 (−), 36.5 (+), 25.8 (+), 17.8 (+), 16.6 (+).

(2 RS*,3*S*)-3,6-Dimethylhept-5-en-2-ol (**15**).* A solution of 3.6 g (46.5 mmol) dimethylsulfoxide in 10 ml dry dichloromethane was added dropwise to a −70°C cold, vigorously stirred solution of 2.92 g (23.0 mmol) oxalylchloride in 80 ml dry dichloromethane. After stirring the solution for 20 min, a solution of 1.95 g (15.3 mmol) of the alcohol **14** in 5 ml dichloromethane was added. After stirring for another hour at −70°C, 14.8 g (91.8 mmol) triethylamine were added. During the following hour, the mixture was allowed to warm to 0°C and subsequently poured on 150 ml ice water. After separation of the layers, the aqueous layer was extracted 3 times with portions of 50 ml diethylether. The combined organic solutions were washed with diluted hydrochloric acid, a saturated solution of aqueous hydrogencarbonate, and brine. The solution of the obtained (*S*)-2,5-dimethylhex-4-enal was dried over magnesium sulfate, carefully concentrated *in vacuo*, and immediately used for the next step.

^{1}*H NMR (400 MHz; CDCl$_3$)*: δ [ppm]; J(Hz): 9.65 (1H, d, H-1), 5.12–5.07 (1H, m, H-4), 2.44–2.32 (2H, m, H-3), 2.18–2.07 (1H, m, H-2), 1.70 (3H, s, Me-5), 1.62 (3H, s, Me-5′), 1.08 (3H, d, Me-2). $J_{1,2} = 1.5$, $J_{2,\mathrm{Me}} = 6.6$.

^{13}*C NMR (101 MHz, CDCl$_3$, DEPT)*: δ [ppm]: 205.3 (C-1), 134.1 (C-5), 120.6 (C-4), 46.8 (+), 29.1 (C-3), 17.9 (+), 15.3 (+), 13.1 (+).

To a −78°C cold solution of 80 mg (635 μmol) of freshly prepared (*S*)-2,5-dimethylhex-4-enal in 2 ml dry tetrahydrofuran, was added 1.5 mmol methyllithium (1.5 ml of a 1 M solution in hexane). The solution was warmed to room temperature and poured on a mixture of 20 ml ice water and 20 ml diethylether. After separation of the layers, the aqueous layer was extracted 3 times with portions of 20 ml of diethylether. The combined organic solutions were washed with brine, dried over magnesium sulfate, and concentrated. The crude product was purified by column chromatography to yield 60 mg (415 μ mol = 64%) of the diastereomers (2*RS*,3*S*)-3,6-dimethylhept-5-en-2-ol, (**15)**.

^{1}*H NMR (400 MHz; CDCl$_3$)*: δ [ppm]; J(Hz): 5.15 (m, 1H, H-5), 3.72 (m, 0.6H, H-2), 3.65 (m, 0.4H, H-2), 2.12 (m, 1H, H-4), 1.88 (m, 1H, H-4), 1.71 (s, 3H, Me-6), 1.61 (s, 3H, C-7), 1.55 (m, 1H, H-3), 1.17 (d, 0.6x3H, Me-1), 1.15 (d, 0.4 × 3H, Me-1), 0.89 (d, 0.6 × 3H, Me-3), 0.88 (d, 0.4 × 3H, Me-3).

^{13}*C NMR (101 MHz, CDCl$_3$):* δ [ppm]: mixture of diastereomers, all visible signals are listed: 132.6 (C6), 123.1 (C5), 71.8 (C2), 71.0 (C2), 41.0 (C3), 40.4

(C3), 31.5 (C4), 25.9 (C-Me-6), 20.4 (C1), 19.8 (C1), 17.8 (C7), 15.1 (Me-3), 14.0 (Me-3).

Following the transformation of **9** into **15**, the same sequence was started from methyl (*S*)-3-hydroxy-2-methylpropanoate to produce the complementary pair of diastereomers, (3*R*,4*RS*)-3,6-dimethylhept-5-en-2-ol, showing the same spectroscopic data as **15**.

*Transformation of Unsaturated Precursors into 1,4-Di(Trifluoroacetoxy)-3-Methylpentane, **8**.* A solution of ca. 0.5 mg of the diastereomers (2*RS*,3*S*)-3,6-dimethylhept-5-ene-2-ol, **15**, in 2 ml dichloromethane was transferred to a 5 ml septum-capped vial equipped with an inlet capillary and an outlet. The vial was cooled to -78°C, and a gentle stream of ozone was passed through the solution until it turned blue. Subsequently, pure oxygen was passed through for 10 min, and 10 mg of solid sodium borohydride was added. The suspension was stirred for 2 hr and filtered over cotton. To the clear solution were added 15 mg of trifluoroacetic acid anhydride at 0°C. This mixture was carefully concentrated to dryness, and the residue was dissolved in 50 μl of dichloromethane. This sample was directly analyzed by GC-MS. The transformation of (2*RS*,3*S*)-3,6-dimethylhept-5-ene-2-ol, **15**, into (3*S*,2*RS*)-1,4-ditrifluoroacetoxy)-3-methylpentane, (3*S*,2*RS*)-**8**, proved to be quantitative. The same transformation procedure was carried out with concentrated pentane extracts of Dufour's glands of *G. striatula*.

RESULTS AND DISCUSSION

The gas chromatogram of a pentane extract of Dufour's gland of *G. striatula* showed two major components (Blatrix, et al., 2002). The mass spectrum of the main component **1** revealed a molecular ion at $M^+ = 322$ (Figure 3). The base peak of the spectrum at $m/z = 155$ ($C_{10}H_{19}O$ upon HR-MS) and two very small signals at $m/z = 172$ and $m/z = 173$ (acid and protonated acid) as well as the presence of $m/z = 60$ revealed **1** to be an ester of decanoic acid (Francke et al., 2000). An abundant signal at $m/z = 150$ ($C_{11}H_{18}$ upon HR-MS) representing the alkene fragment corresponding to the alcohol component, suggested the alcohol to be an undecadienol ($m/z = 168$ corresponding to the molecular weight of the alcohol present) with a primary hydroxyl function (Tengö et al., 1985; Francke et al., 2000). Signals at $m/z = 253$ ($M^+ - 69$) and $m/z = 69$ indicated a dimethylallyl substructure, typical for open chain terpenes like geraniol. Considerations on the incorporation of homomevalonate during the biosynthesis of the target terpenoid (Francke and Schulz, 1999) suggested the additional carbon to generate a 3,4,7-trimethyl-2,6-octadiene-1-yl moiety or its 3-ethyl-7-methyl-2,6-octadiene-1-yl isomer.

The mass spectra of **2**, the second major component among the Dufour's gland volatiles and that of a minor compound, **3**, showed molecular ions at $M^+ = 350$

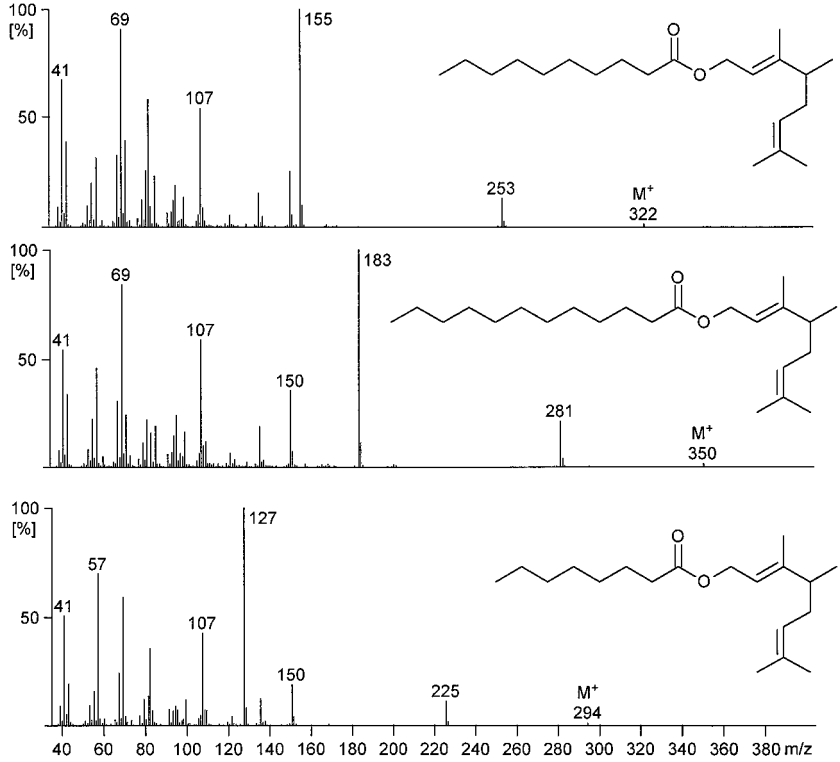


FIG. 3. 70-eV mass spectra of the main volatiles **1–3** in the pentane extract of Dufour's gland of *G. striatula*.

and $M = 294$ as well as base peaks at $m/z = 183$ and $m/z = 127$, respectively, and a similar fragmentation pattern in the lower mass region (Figure 3). This suggested the compounds to be the dodecanoate and the octanoate of the same alcohol component as in ester **1**. Small amounts of esters of this alcohol and other carboxylic acids, e.g., the hexanoate, heptanoate, nonanoate, undecanoate, and tridecanoate were also found (see Blatrix et al., 2002).

Microozonolysis of the natural extract followed by reduction and trifluoroacetylation of the degradation product furnished a compound that perfectly matched the gas chromatographic properties and the mass spectrum of synthetic 1,4-di(trifluoroacetoxy)-3-methylpentane (Figure 2B). This finding revealed the target alcohol to be the homoterpene 3,4,7-trimethyl-2,6-octadiene-1-ol (4-methylgeraniol) and, thus, the natural volatiles to be carboxylic acid esters thereof.

The mass spectrum of another minor constituent of the extract, compound **4**, is shown in Figure 4. The compound eluted later than the major decanoate **1** but

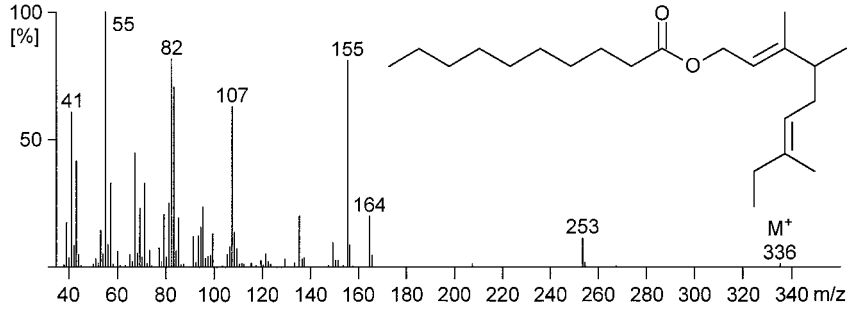


FIG. 4. 70-eV Mass spectrum of compound **4** in the pentane extract of Dufour's gland of *G. striatula.*

also showed a base peak at $m/z = 155$, again suggesting a decanoic acid ester. However, compared to 4-methylgeraniol in **1**, the alcohol component contained an additional carbon atom as indicated by the corresponding alkene fragment at $m/z = 164$ (see Figure 4). Since the spectrum showed only a small signal at $m/z = 69$ but abundant fragments at $m/z = 253$ ($M^+ - 83$) and $m/z = 83$, the additional carbon was clearly attached to the tail of the 4-methylgeraniol. Thus, the alcohol was the bishomogeraniol, 3,4,7-trimethyl-2,6-nonadiene-1-ol. Apart from its decanoate **4**, several other esters of this alcohol were detected (see Blatrix et al., 2002).

Unambiguous proof for the postulated structures was obtained through the synthesis of both (2*E*,6)-3,4,7-trimethyl-2,6-octadiene-1-ol and (2*E*,6)-3,4,7-trimethyl-2,6-nonadiene-1-ol and esterification with the appropriate carboxylic acids (Figure 1). The syntheses of both terpenoid alcohols and their esters were straightforward following a known procedure (Ohnuma et al., 1989). Since that of bishomogeraniol started with an *E*/*Z*-mixture of 1-bromo-3-methyl-2-pentene, a mixture of the 2*E*,6*Z* and 2*E*,6*E* alcohols was obtained. Unambiguous structure assignments of the stereoisomers by NMR-spectroscopy was based on NOE experiments using the methylene groups C-5 and C-8, as well as the methyl group at C-7 of (2*E*,6)-3,4,7-trimethyl-(2,6)-nonadiene-1-ol. The relative proportion of the 2*E*,6*Z* and the 2*E*,6*E* esters synthesized as the final products was 1:1.6. Mass spectra and gas chromatographic retention times of all synthetic esters completely matched those of corresponding natural products.

Since both terpenoids show a stereogenic center, the absolute configuration of the natural products remained to be determined. As mentioned earlier, ozonization of the extract, reductive work-up, and trifluoroacetylation yielded 1,4-di(trifluoroacetoxy)-3-methylpentane. A synthetic mixture containing all four stereoisomers was perfectly separated upon GC using a modified cyclodextrin as the stationary phase (Figure 5, trace a).

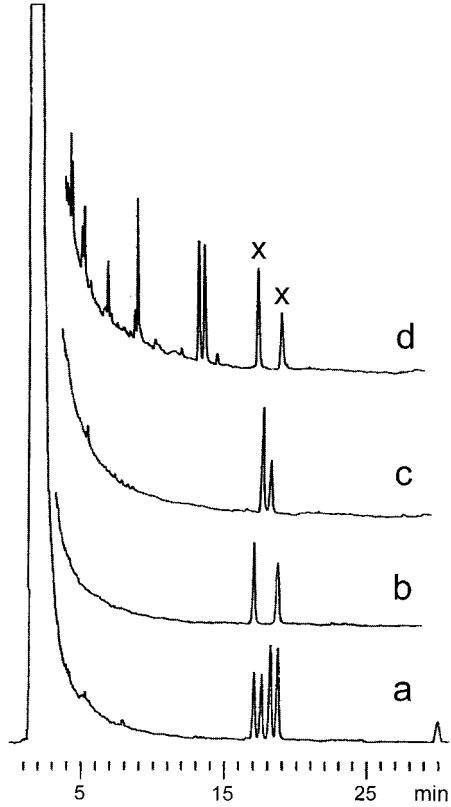


FIG. 5. Gas chromatographic separation of the four stereoisomers of 1,4-di (trifluoroacetoxy)-3-methylpentane (trace a), (3*S*,4*RS*)-1,4-di(trifluoroacetoxy)-3-methylpentane (trace b)[1] (3*R*,4*RS*)-1,4-di(trifluoroacetoxy)-3-methylpentane (trace c), and the 1,4-di(trifluoroacetoxy)-3-methylpentane (trace d) obtained after ozonization/derivatization of Dufour's gland extract. For conditions see text.

Upon ozonization/derivatization, the two series of esters of 4-methylgeraniol and bishomogeraniol furnish the same product, 1,4-di(trifluoroacetoxy)-3-methylpentane, **8**. During this procedure, an enantiomerically pure homoterpene yielded one pair of diastereomers since the second stereogenic center at C-4 of the derivative is introduced through the degradation process. Indeed, after derivatization, the natural extract produced only two peaks on the chiral stationary phase at the relevant retention time (Figure 5, trace d marked with $\times$s) proving the natural products to be enantiomerically pure. Consequently, samples of **8** with a defined configuration at the methyl branching were needed for the assignment of the absolute configuration of 1,4-di(trifluoroacetoxy)-3-methyl pentane derived from the

natural product through comparison of gas chromatographic retention times with authentic reference compounds.

Our synthesis of these substances started from commercially available methyl (*S*)-3-hydroxy-2-methylpropanoate **9** (Figure 2B), which was protected at the hydroxy group to furnish the ester **10**. This was reduced to **11** and converted to (*S*)-4-methoxymethoxy-3-methylbutyronitrile, **12**, through a conventional sequence. Reduction of the nitrile to the corresponding aldehyde with diisobutylaluminium hydride and Wittig reaction with isopropyl triphenylphosphonium iodide yielded (*S*)-1-methoxymethoxy-3,6-dimethylhex-4-ene, **13**. Deprotection, Swern oxidation of the resulting primary alcohol, and reaction with methyl lithium provided a mixture of diastereomers, (2*RS*,3*S*)-3,6-dimethylhept-5-ene-2-ol, **15**.

This product, which we also used in the synthesis of homoterpenes (Lehmann and Francke, unpublished), was submitted to the previously described ozonization/derivatization. Upon gas chromatography on the cyclodextrin column, the obtained (3*S*,4*RS*)-1,4-di(trifluoroacetoxy)-3-methylpentane produced two peaks, which matched two of the four peaks obtained with the mixture of all four stereoisomers, generated from racemic 4-methylgeraniol. Correspondingly, the remaining two peaks of the mixture of stereoisomers matched the retention times of (3*R*,4*RS*)-1,4-di(trifluoroacetoxy)-3-methylpentane (Figure 5, trace b and c) derived from (3*R*,2*RS*)-3,6-dimethylhept-5-ene-2-ol.

As seen in Figure 5, trace d, ozonization/derivatization of crude Dufour's gland extract yielded two peaks that coincided with those produced by (3*R*,4*RS*)-1,4-di(trifluoroacetoxy)-3-methylpentane (Figure 5, trace c). The 3*R*,4*RS*-stereoisomers (Figure 5, trace c) could not be observed (detection limit 1–2%). This proves that at least all esters of 4-methylgeraniol and of bishomogeraniol present in amounts of $>1\%$ of the Dufour's gland volatiles show (4*S*)-configuration.

Esters of monoterpene alcohols with straight chain fatty acids like geranyl octanoate have been identified as volatiles of several bee species (Bergström and Tengö, 1974), and they also occur in other organisms. However, esters of C_{11} or C_{12} homoterpenes represent a new class of natural products. The biosyntheses of the two new alcohols may involve the incorporation of one or two homomevalonate units, respectively (Figure 6) (Francke and Schulz, 1999). This is supported by the fact that (*S*)-4-methylgeraniol has been enzymatically prepared through the action of a farnesyl pyrophosphate synthetsase on a mixture of 3-methyl-2-butene-1-yl diphospate and (*E*)-3-methyl-3-pentene-1-yl diphosphate (Koyama et al., 1980). The new terpenoid alcohols show the same vicinal dimethyl branching as faranal (3*S*,4*R*,6*E*,10*Z*)-3,4,7,11-tetramethyl-6-10-tridecadienal, the trail pheromone of the pharaoh's ant, *Monomorium pharaonis* (Kobayashi et al., 1980). The same branching pattern is also present in (3*E*,6*E*,10*Z*)-3,4,7,11-tetramethyl-1,3,6,10-tridecatetraene, one of the trail pheromone components of the fire ant, *Solenopsis invicta* (Alvarez et al., 1987). Homofarnesenes and bishomofarnesenes have

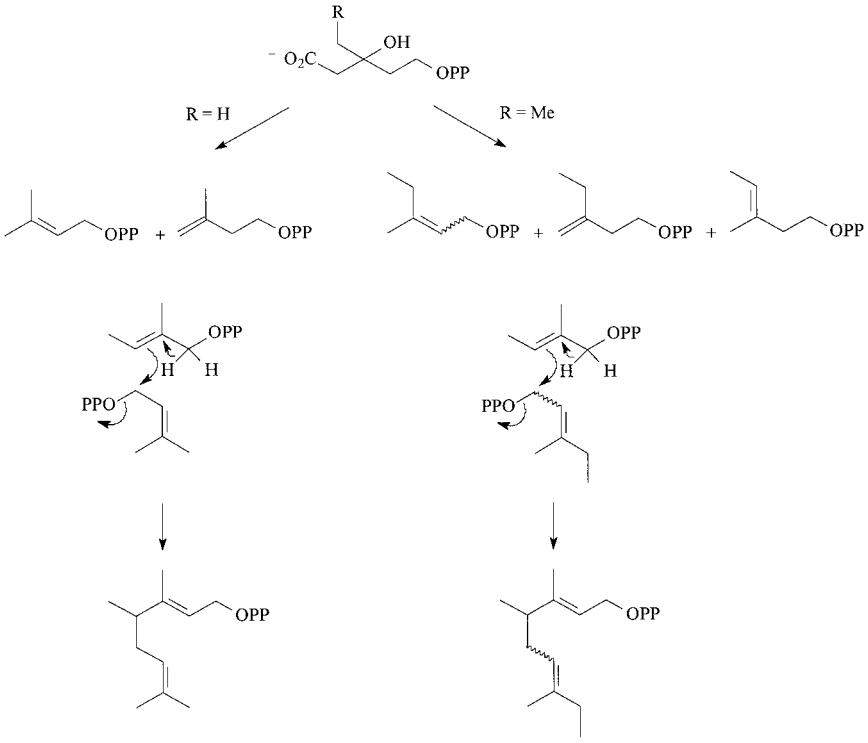


FIG. 6. Possible biosynthesis of 4-methylgeraniol and bishomogeraniol.

been repeatedly reported as constituents of Dufour's gland secretions of *Myrmica* ants (Morgan et al., 1979; Cammaerts et al., 1981; Attygalle and Morgan 1982; Attygalle et al., 1983). However, the structures of these compounds show ethyl branching instead of geminal dimethyl branching, which points to a slightly different biosynthesis from homomevalonate. Interestingly, methyl (4*S*,10*R*,11*S*,2*E*, 6*E*)-10,11-epoxy-7-ethyl-3,4,11-trimethyl-2,6-tridecadienoate [(+)-4-methyl juvenile hormone I], possibly formed from three homomevalonate units, shows a vicinal dimethyl substructure as well as internal ethyl branching.

Elucidation of the biological significance of the main terpenoid esters identified in Dufour's gland of *G. striatula* is described in the following paper (Blatrix et al., 2002).

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TRAIL PHEROMONE OF PONERINE ANT *Gnamptogenys striatula*: 4-METHYLGERANYL ESTERS FROM DUFOUR'S GLAND

RUMSAÏS BLATRIX,[1] CLAUDIA SCHULZ,[2] PIERRE JAISSON,[1] WITTKO FRANCKE,[2] AND ABRAHAM HEFETZ[3,*]

[1]*Laboratoire d'Ethologie Expérimentale et Comparée (FRE CNRS 2413)*
Université Paris Nord
93430 Villetaneuse, France

[2]*Institut für Organicshe Chemie, Universität Hamburg*
Martin Luter King Platz 6, D-20146 Hamburg, Germany

[3]*Department of Zoology, George S. Wise Faculty of Life Sciences*
Tel-Aviv University
Ramat Aviv 69978, Israel

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Abstract—Dufour's gland is the origin of the trail pheromone of *Gnamptogenys striatula*. Chemical analysis of the glandular extracts revealed a series of new natural products, especially esters of (2*E*)-3,4,7-trimethyl-2,6-octadien-1-ol (4-methylgeraniol), and (2*E*)-3,4,7-trimethyl-2,6-nonadien-1-ol (a bishomogeraniol isomer) with medium-chain fatty acids. Bioassays with synthetic racemates of the esters revealed that the 4-methylgeranyl esters are highly active as trail pheromones, while the bishomogeranyl esters are either marginally active or not active at all. Assays with the individual 4-methylgeranyl esters showed each of them to be inferior to the glandular secretion in eliciting trail following. However, the mixture of racemic 4-methylgeranyl octanoate and the corresponding decanoate and dodecanoate, the main Dufour's volatile constituents, is as active as the natural secretion at similar concentration. We conclude that the trail pheromone constitutes a mixture of at least the 4-methylgeranyl esters identified in the gland. Since *G. striatula* generally preys on small arthropods rather than monopolizing large resources, we assume that trails are rarely used during foraging, but more often during nest migration. Production of new societies in this species is generally performed by budding, a period of considerable predation risk. Utilizing trails for efficient displacement in this context is, therefore, highly

* To whom correspondence should be addressed. E-mail hefetz@post.tau.ac.il

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adaptive. This behavioral repertoire may also provide the ants with additional means of food resource exploitation.

Key Words—*Gnamptogenys*, Dufour's gland, trail pheromones, 4-methylgeranyl esters.

INTRODUCTION

Mass recruitment of conspecifics to a food source constitutes a complex process that starts by alert behavior performed by scout ants, inducing the surrounding ants to exit the nest. Subsequently, these ants generally follow the chemical trail previously laid by the scout ant during its return from the food source. The information encoded in trail pheromones often constitutes several signals, such as excitatory (recruitment) cues that induce the ants to leave the nest as well as follow the trail, orientation cues that direct the ants to the food source, and often synergistic compounds that enhance recruitment. Ants use a variety of glands encompassing a plethora of compounds to mark their trails. Therefore, it is hard to predict which gland is used in a given species (Hölldobler and Wilson, 1990).

The trail pheromones of only a few ponerine ants have been disclosed. Most species utilize the poison gland exudate to mark the trail and often the pygidial gland for recruiting and inducing potential foragers to exit the nest. For example, in *Pachycondyla (Megaponera) foetens*, actinidine from the pygidial gland stimulates the ants to forage, while *N*,*N*-dimethyl uracil from the poison gland releases trail-following behavior (Janssen et al., 1995). Ponerine poison gland products can also be made up by propanoate units or originate from a mixed acetate–propanoate biosynthesis (Francke and Schulz, 1999). For example, *Leptogenys diminuta* uses (3*R*, 4*S*)-4-methyl-3-heptanol as a single trail pheromone component (Attygalle et al., 1988), whereas *L. peuqueti* uses a complex mixture of acetates and alcohols (Janssen et al., 1997). *Ectatomma ruidum* and *Gnamptogenys menadensis* are unusual among the ponerine ants investigated to date in employing Dufour's gland secretion for trail marking. All-*trans* geranylgeranyl acetate, the major component in the secretion of *E. ruidum*, is the most active, accompanied by the less active corresponding alcohol geranylgeraniol (Bestmann et al., 1995). Dufour's gland secretion of *G. menadensis* is typified by a mixture of straight-chain hydrocarbons and terpenes, with heptadecane and bishomo-β-farnesene as major components, respectively. Whether or not all of the glandular components or specific compounds comprise the active trail pheromone was not determined (Gobin et al., 1998).

The ponerine ant *Gnamptogenys striatula* belongs to the tribe Ectatommini and exhibits a derived social structure, similar to that of some "higher" ants (Blatrix, 2000). Colonies can contain up to 1200 workers, a large number for a ponerine, and 60 queens, about 60% of which are functional, resulting in one of the highest degrees of polygyny in ants (Blatrix and Jaisson, 2001). Most *Gnamptogenys*

species feed on small arthropods, and *G. striatula* is known to be a generalist predator (Lattke, 1995). Sweet liquids weakly attract workers, and predation on large arthropods (i.e., requiring several workers for transportation) has been observed only occasionally in the field (Blatrix, personal observation).

In this study, we investigated the glandular origin and chemical nature of the trail pheromone in *G. striatula*.

METHODS AND MATERIAL

Ants. Colonies of *G. striatula* were collected near Itabuna (Bahia, Brazil) and reared in the laboratory in artificial nests made of plaster of Paris. Extracts of various body parts were obtained by cutting frozen ants and crushing the appropriate body parts in pentane filled vials. Glandular extracts were obtained by dissecting frozen ants in distilled water under a stereomicroscope and placing the appropriate gland in pentane (0.1 body part or gland equivalents per microliter).

Chemical Analyses. For chemical analyses, pentane extracts were concentrated and analyzed by coupled gas chromatography mass spectrometry: A Hewlett-Packard gas chromatograph 5890 (Palo Alto, California, USA) was linked to a mass spectrometer VG 70/250 SE (Vacuum Generators, Manchester, UK) (Klimetzek et al., 1989). With helium as the carrier gas, separations were achieved with a 60-m $\times$ 0.25-mm-ID, 0.25-μm film DB5-MS fused silica column (J & W Scientific, Folsom, California, USA) under the following conditions: 2 min at 50°C, then programmed to 280°C at a rate of 5°C/min.

Quantification of Dufour's gland content was done by gas chromatography using pools of 10 glands extracted in pentane containing 100 ng of octadecane as an internal standard. The samples were run on a Hewlett-Packard gas chromatograph equipped with a DB-1 capillary column, temperature programmed from 110°C (1 min hold) to 300°C at 10°C/min. The total amount of glandular content was calculated by peak integration as compared to the internal standard.

Synthetic reference samples of racemic (2*E*,6)-3,4,7-trimethyl-2,6-octadien-1-ol (4-methylgeraniol) and (2*E*,6)-3,4,7-trimethyl-2,6-nonadien-1-ol, as well as corresponding fatty acid esters, were prepared according to known methods (Ohnuma et al., 1989; Schulz et al., 2002)

Bioassays. Artificial trails were made with 20 μl of the test solution applied on one arm of an ellipse (20 cm long) drawn on a sheet of paper (0.1 part or gland/μl). The other arm was applied with the control solution. A short trail before and after each ellipse was made with abdominal extracts (0.1 abdomen equivalents/cm; trail length 5 cm) because preliminary studies revealed that extracts of this body part induced the best trail-following behavior. Trails were applied 10 min before each test to allow the solvent and alarm eliciting substances to evaporate (unpublished data). For some tests, a circular area was marked (2.5 cm diam.) at the bifurcation point to assess the number of ants that congregated at that point before selecting

between the arms of the ellipse (decision circle). Adult dead *Drosophila* were provided at the end of the trail, but to prevent trail overmarking by returning ants, all the ants that held a prey between the mandibles were immediately aspirated. For each test, two successive ellipses were drawn creating two bifurcation points. Assignment of the arms (test or control) in each ellipse was at random.

Before each test, all foraging ants were confined within the nest and the nest entrance blocked until the beginning of the test. Trail following was measured by recording the number of ants on each arm of the ellipse and in the "decision circle" ("hesitating ants") every 15 sec during a 10-min test. The cumulative number (mean $\pm$ SD) of ants in each of the ellipse arms and the mean proportion of hesitating ants (= number of hesitating ants/total number of recorded ants during the test) in the decision circle served for scoring trail-following activity. The numbers of ants on each arm were compared with a Wilcoxon matched-pairs test.

RESULTS

In the preliminary tests, the ability of different body parts (head, alitrunk, and abdomen) to elicit trail following was tested against a pentane control. Since in all cases the ants preferred and followed the body part extracts over pentane, we modified the assay and tested the various body parts against each other (Table 1, tests 1 and 2). Abdominal extracts were more active than those from alitrunk or

TABLE 1. TRAIL-FOLLOWING RESPONSE OF WORKERS TO BODY PARTS OR GLANDULAR EXTRACTS[a]

Test	Extract	Cumulative number of ants on trail (mean $\pm$ SD)	*N*	*P*
1	Abdomen	22 ± 8	12	<0.01
	Alitrunk	7 ± 3		
2	Abdomen	45 ± 24	12	<0.01
	Head	13 ± 12		
3	Alitrunk	31 ± 18	12	<0.01
	Dufour's gland	62 ± 18		
4	Alitrunk	41 ± 19	12	NS
	Poison gland	33 ± 20		
5	Alitrunk	49 ± 24	12	<0.01
	Pygidial gland	13 ± 10		
6	Abdomen	58 ± 20	10	NS
	Dufour's gland	49 ± 15		
7	Poison + Dufour's glands	75 ± 23	12	<0.01
	Rest of the abdomen	46 ± 11		
8	Poison + Dufour's glands	48 ± 19	12	NS
	Dufour's gland	55 ± 23		

[a] The mean numbers of ants on each arm of the ellipse were compared by a Wilcoxon matched pairs test (N = number of tests performed).

heads. In the next set of tests, the activity of various abdominal glandular secretions was compared to the alitrunk extract. Only Dufour's gland was more active than the alitrunk, but the glandular secretion was still less active than the whole abdominal extracts (Table 1, tests 3–6). To assess whether more than one gland is involved in trail signaling, combined extracts of poison and Dufour's glands were compared to the rest of the abdomen. The combined glandular extract was more active than the whole abdominal extract. However, when the combined Dufour's–poison glands extract was tested against Dufour's gland extract alone, there was no preference between the two alternative trails (Table 1, tests 7 and 8). Thus, Dufour's gland secretion accounts for all the trail pheromone activity, and there is no synergism with the content of the poison gland.

Quantification of Dufour's gland secretion, based on pools of 10 glands, revealed an average of 173.1 ± 70 ng volatile constituents per gland. Chemical analysis of the glandular content by combined gas chromatography/mass spectrometry (GC-MS) showed the presence of a multicomponent mixture, dominated by esters of (2*E*,6)-3,4,7-trimethyl-2,6-octadien-1-ol (4-methylgeraniol) (Schulz et al., 2002). This homologous series of esters ranged from traces of the hexanoate to tridecanoate (including one decenoate and two dodecenoates), with the octanoate, the decanoate, and the dodecanoate esters as the major components (Figure 1, Table 2). A second series, comprising minute amounts of the octanoate,

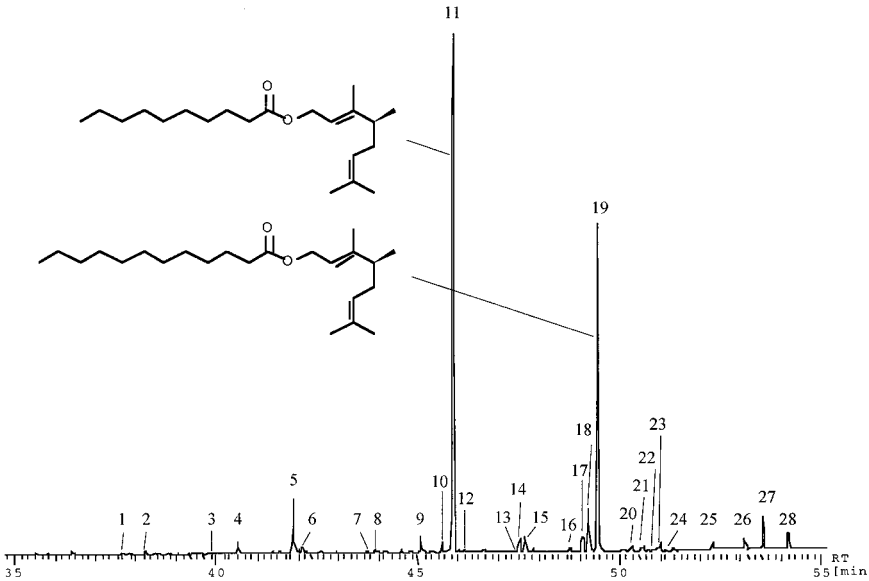


FIG. 1. Gas chromatogram of a pentane extract of Dufour's gland of *G. striatula* workers obtained with a non-polar column. Peak numbers are the same as in Table 2. For conditions see Methods and Materials.

TABLE 2. CHEMICAL COMPOSITION OF DUFOUR'S GLAND OF *G. striatula* WORKERS[a]

Peak	Compound	Relative amounts
1	(4S,2E)-3,4,7-Trimethyl-2,6-octadienyl hexanoate (ct)	t
2	Unknown terpenoid	t
3	(4S,2E)-3,4,7-Trimethyl-2,6-octadienyl heptanoate (ct)	t
4	Unknown terpenoid	t
5*	(4S,2E)-3,4,7-Trimethyl-2,6-octadienyl octanoate	8.8 ± 1.8
6	Octadecenal	t
7	(4S,2E,6E)-3,4,7-Trimethyl-2,6-nonadienyl octanoate (ct)	t
8	(4S,2E)-3,4,7-Trimethyl-2,6-octadienyl nonanoate	2.5 ± 0.1
9	Geranyl decanoate	3.0 ± 0.3
10	(4S,2E)-3,4,7-Trimethyl-2,6-octadienyl decenoate (ct)	t
11*	(4S,2E)-3,4,7-Trimethyl-2,6-octadienyl decanoate	52.9 ± 1.7
12	Eicosenal	t
13	(4S,2E,6Z)-3,4,7-Trimethyl-2,6-nonadienyl decanoate (ct)	t
14*	(4S,2E,6E)-3,4,7-Trimethyl-2,6-nonadienyl decanoate	1.1 ± 0.1
15	(4S,2E)-3,4,7-Trimethyl-2,6-octadienyl undecanoate (ct)	1.0 ± 0.1
16	Geranyl dodecanoate	t
17	(4S,2E)-3,4,7-Trimethyl-2,6-octadienyl dodecenoate (ct)	1.2 ± 0.2
18	(4S,2E)-3,4,7-Trimethyl-2,6-octadienyl dodecenoate	3.9 ± 0.7
19*	(4S,2E)-3,4,7-Trimethyl-2,6-octadienyl dodecanoate	22.3 ± 1.6
20	Octanoate of unknown terpenoid	t
21	Unknown terpenoid	t
22	(4S,2E,6Z)-3,4,7-Trimethyl-2,6-nonadienyl dodecanoate (ct)	t
23*	(4S,2E,6E)-3,4,7-Trimethyl-2,6-nonadienyl dodecanoate	3.5 ± 0.9
24	(4S,2E)-3,4,7-Trimethyl-2,6-octadienyl tridecanoate	t
25	Unknown terpenoid	t
26	Decanoate of unknown terpenoid	1.0 ± 0.1
27	Decanoate of unknown terpenoid	3.8 ± 1.1
28	Decanoate of unknown terpenoid	1.3 ± 0.6

[a] Peak numbers correspond to those in Figure 1. Racemates of compounds marked with an asterisk constitute part of the synthetic mixture in Table 3. ct = Assignment of absolute configuration is tentative, t = trace.

the decanoate, and the dodecanoate of (2*E*,6)-3,4,7-trimethyl-2,6-nonadien-1-ol was also identified. Careful GC-MS analyses using synthetic reference samples proved the esters of the 2*E*,6*E* alcohol to be accompanied by even smaller amounts of the earlier eluting 2*E*,6*Z* stereoisomers. In addition, small amounts of geranyl decanoate and geranyl dodecanoate, octanoate, and decanoate esters of unknown terpenoid alcohols, unidentified terpenoids, and two straight chain aldehydes, octadecenal and eicosenal were identified in the secretion.

Synthetic racemates of the three major 4-methylgeranyl esters and two of the bishomogeranyl esters (Schulz et al., 2002; marked with an asterisk in Table 2) were bioassayed as described above. A mixture of the five esters at a concentration of 200 μg/ml each (total of 1 μg/cm trail) was more active than pentane (blank), and

TABLE 3. TRAIL-FOLLOWING RESPONSE OF ANTS TO PENTANE SOLUTIONS OF BLENDS OF FIVE SYNTHETIC TERPENOID ESTERS AND TO PENTANE EXTRACTS OF DUFOUR'S GLAND[a]

Test	Extract	Proportion of hesitating ants[b]	Cumulative number of ants on trail (mean ± SD)	*N*	*P*
1	Synthetic mixture (1 μg/cm)		76 ± 67	20	<0.01
	pentane		2 ± 2		
2	Synthetic mixture (1 μg/cm)		53 ± 60	12	NS
	Dufour's gland (0.1 GE/cm)		64 ± 61		
3	Synthetic mixture (10 ng/cm)	0.50	55 ± 15	10	<0.01
	pentane		2 ± 2		
4	Synthetic mixture (4 ng/cm)	0.78	11 ± 7	10	<0.01
	pentane		1 ± 1		
5	Synthetic mixture (2 ng/cm)	0.97	3 ± 2	2	NS
	pentane		1 ± 1		
6	Synthetic mixture (1 ng/cm)	0.94	3 ± 2	4	NS
	pentane		1 ± 2		

[a] Mean number of ants on each trail were compared by a Wilcoxon matched pairs test (N = number of tests performed). GE = gland equivalents NS = non significant.
[b] For definition, see Methods and Materials.

induced trail following by the foragers. Moreover, there were no differences in trail following between the synthetic mixture and extracts of Dufour's gland (Table 3, tests 1 and 2). In order to determine the active threshold of the mixture, serial dilutions (1/100, 1/250, 1/500, 1/1000) of the original mixture were tested against pentane. At 1/250 dilution (4ng/cm trail), the synthetic mixture still induced trail-following behavior, but the number of ants that followed the trail was considerably reduced. On the other hand, a great proportion of the ants (0.78) congregated inside the circle drawn at the bifurcation point. Both parameters indicated that this dilution was nearly at the threshold concentration for response (Table 3). At lower concentrations, the ants were not able to detect the synthetic trail mixture. The number of ants in the test arm was small and not different from the control arm, and the proportion of hesitating ants increased considerably (1/500: 0.97, 1/1000: 0.94).

Tests with individual synthetic esters revealed that the 4-methylgeranyl esters were all active, with large numbers of ants following the synthetic trail and almost none the pentane applied arm (Table 4, tests 1–3). Of the bishomogeranyl esters, the decanoate was totally inactive (Table 4, test 4), whereas the dodecanoate ester was significantly more active than pentane (Table 4, test 5). However, in the latter case, the cumulative number of ants on the trail was low. There were no differences in the proportion of hesitating ants when tested with either of the bishomogeranyl esters, and in both cases the number was considerably higher when compared to any of the 4-methylgeranyl esters.

To verify that only the 4-methylgeranyl esters are active, two alternative trails, one containing 4-methylgeranyl decanoate and the second bishomogeranyl

TABLE 4. TRAIL-FOLLOWING RESPONSE OF ANTS TO PENTANE SOLUTIONS OF PURE RACEMATES OF SYNTHETIC ESTERS AND TO PENTANE EXTRACTS OF DUFOUR'S GLAND[a]

Test	Test solution	Proportion of hesitating ants[b]	Cumulative number of ants on trail (mean ± SD)	N	P
1	4-Methylgeranyl octanoate, 10 ng/cm (5)	0.38	112 ± 100	6	<0.05
	pentane		1 ± 1		
2	4-Methylgeranyl decanoate, 10 ng/cm (11)	0.60	23 ± 12	6	<0.05
	pentane		1 ± 1		
3	4-Methylgeranyl dodecanoate, 10 ng/cm (19)	0.66	18 ± 14	12	<0.01
	pentane		2 ± 1		
4	Bishomogeranyl decanoate, 100 ng/cm (14)	0.86	2 ± 2	8	NS
	pentane		2 ± 1		
5	Bishomogeranyl dodecanoate, 100 ng/cm (23)	0.85	5 ± 3	10	<0.01
	pentane		1 ± 1		
6	4-Methylgeranyl decanoate, 10 ng/cm	0.41	46 ± 17	6	<0.05
	Bishomogeranyl decanoate, 100 ng/cm		1 ± 1		
7	4-Methylgeranyl octanoate, 10 ng/cm		12 ± 6	6	<0.05
	Dufour's gland, 0.1 GE/cm =~17.3 ng/cm		73 ± 41		
8	4-methylgeranyl decanoate, 10 ng/cm		30 ± 9	6	<0.05
	Dufour's gland, 0.1 GE/cm		52 ± 17		
9	4-Methylgeranyl dodecanoate, 10 ng/cm		11 ± 4	6	<0.05
	Dufour's gland, 0.1 GE/cm		66 ± 23		
10	Mixture of 5 + 11 + 19, 10 ng/cm		27 ± 14	6	NS
	Dufour's gland, 0.1 GE/cm		35 ± 19		

[a] Numbers in parentheses refer to peak numbers in Figure 1 and Table 2. Mean number of ants on each trail were compared by a Wilcoxon matched pairs test (N = number of tests performed). NS = Not significant.
[b] For definition, see materials and methods.

decanoate, were offered to the ants. The ants followed the trail of 4-methylgeranyl decanoate without hesitation (Table 4, test 6). Further tests aimed at assessing whether the single synthetic compounds found to be most active (products 5, 11, and 19 in Table 2) are as active as the glandular secretion. Dufour's gland was superior to any of the three 4-methylgeranyl esters tested (Table 4, tests 7–9), but the mixture of all three products at a concentration of 10 ng/cm trail was as active as Dufour's gland secretion (Table 4, test 10).

DISCUSSION

Ponerine ants are known to use visual cues and tandem running as orienting systems, while species using trail pheromone are far less common. Trail strategy in ponerine ants seems to occur in three contexts: foraging on immobile and large quantities of resources, species with army-ant-like behavior, and colony migration.

In the case of foraging, species specialized on termite predation or nectar exploitation show well-defined trail-following behavior (Longhurst et al., 1979; Maschwitz and Schonegge, 1983; Hölldobler, 1984; Breed and Bennett, 1985). *Leptogenys diminuta* displays army ant habits and uses trails to conduct raids (Attygalle et al., 1988; Maschwitz et al., 1989), while other *Leptogenys* species use trails during colony migration. There are not enough field studies of *G. striatula*, to determine unequivocally when trails are mostly used. Although in our bioassay we tested trail following in a foraging context, this does not necessarily indicate that the ants use it in that context in the field. It is possible that ants reacted to the signal in a context-independent manner. On the other hand, since these ants are generalist predators of arthropods (Lattke, 1995) and rarely monopolize immobile and large quantities of resources or exhibit any army-ant-like behavior (Blatrix personal observation), we hypothesized that their use of trail pheromones in a foraging context should be rare. We believe that trail deposition in this species is largely linked to frequent nest moving, as was also suggested for *G. menadensis* (Gobin et al., 1998). Colonies are known to perform budding and frequent migrations (Giraud et al., 2000), which seem to be important traits of the life history of this species. Moreover, budding is probably the only way for colonies to multiply. Movement of the colony during budding or migration is risky because of predation, and it is expected to be efficient. We, therefore, hypothesize that orientation by a trail pheromone, rather than tandem running, has evolved in this species in relation to its particular life history.

Our findings demonstrate that Dufour's gland is the origin of the trail pheromone in *G. striatula*. This gland was also shown to be the source of trails deposited by *G. menadensis* and *E. ruidum* (Bestmann et al., 1995; Gobin et al., 1998), all of which belong to the tribe Ectatommini. Other species belonging to the subfamily Ponerinae, on the other hand, use poison and pygidial gland secretion for trail communication. This may indicate a phylogenetic trend in glandular usage, although the number of species studied so far is too limited for generalization. None of the other abdominal glands showed comparable activity. We also could not detect any synergism in trail following between the Dufour's and poison glands.

Chemical analysis of the glandular secretion revealed it to be dominated by series of esters of medium-chain fatty acids with either 4-methylgeraniol or bishomogeraniol. Despite being a rather complex mixture, three esters of 4-methylgeraniol, i.e., the octanoate, the decanoate, and the dodecanoate, comprised about 85% of the total secretion. The chemistry of Dufour's gland in *G. menadensis* differs from that of *G. striatula*, emphasizing the disparate chemical evolution of pheromones that took place in ants. While that of *G. menadensis* is equally rich in linear hydrocarbons and terpenes (Gobin et al., 1998), that of *G. striatula* is almost exclusively composed of terpenoid esters. The chemical disparity between the two *Gnamptogenys* species is not entirely surprising because, although they belong to a single genus, they are not phylogenetically closely related. Furthermore,

G. striatula is a South American species, whereas *G. menadensis* originates in Southeast Asia. Nonetheless, there seems to be consistency in the fact that Dufour's gland constituents represent acetogenines and/or mevalogenines, since the compounds of *G. striatula* represent an interesting combination of these two structural principles.

To ascertain the biological activity of the new natural products identified in Dufour's gland of *G. striatula* and further investigate the possible involvement of particular compounds, synthetic racemates of the three major 4-methylgeranyl and two of the bishomogeranyl esters were tested for trail pheromone activity. There was a clear difference in the activity of the two types of compounds. While the 4-methylgeranyl esters were highly active, bishomogeranyl decanoate was devoid of activity, and bishomogeranyl dodecanoate was only marginally active. It is worth noting that the bishomogeranyl esters were only minor or trace constituents in the secretion and perhaps can be regarded as by-product of the biosynthesis of the 4-methylgeranyl esters or as components of fine tuning in interspecific discrimination. The additional methyl group in bishomogeranyl esters lowered and even abolished trail pheromone activity, underlining the specificity of ant reactions to the active compounds.

From the results obtained with single compounds, it became evident that the pheromone represents a multicomponent mixture. While all of the single active substances were inferior to Dufour's gland secretion, the mixture, even at a lower concentration than the natural Dufour's gland constituents, was as active as the glandular exudates. Since the racemic mixture proved to be active while the natural compounds are represented by almost pure 4*S* enantiomers (Schulz et al., 2002), there are strong indications that, in contrast to many other chiral pheromones, the nonnatural antipode is either biologically inactive or that the configuration at the stereogenic center plays a minor role in trail pheromone activity. Similar phenomena are also known from other chiral semiochemicals and have been carefully reviewed (Mori, 1998). It is still unclear whether the different components delineate different information, or whether those facilitate trail specificity. More studies with closely related species from the same geographic region, as well as studies on the foraging and budding behaviors of *G. striatula* in the field are needed to clarify this point.

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CUTICULAR HYDROCARBONS OF *Tetramorium* ANTS FROM CENTRAL EUROPE: ANALYSIS OF GC-MS DATA WITH SELF-ORGANIZING MAPS (SOM) AND IMPLICATIONS FOR SYSTEMATICS[1]

FLORIAN M. STEINER,[2,*] BIRGIT C. SCHLICK-STEINER,[2] ALEXEJ NIKIFOROV,[3] ROLAND KALB,[3] and ROBERT MISTRIK[4]

[2]*Institute of Zoology, University of Agricultural Sciences*
Gregor Mendelstr. 33, A-1180 Vienna, Austria

[3]*Institute of Organic Chemistry, University of Vienna*
Währingerstr. 38, A-1090 Vienna, Austria

[4]*HighChem Ltd.*
Jana Stanislava 35, 84 105 Bratislava, Slovakia

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Abstract—Cuticular hydrocarbons were extracted from workers of 63 different nests of five species of *Tetramorium* ants (Hymenoptera: Formicidae) from Austria, Hungary, and Spain. The GC-MS data were classified (data mining) by self-organizing maps (SOM). SOM neurons derived from primary neuron separation were subjected to hierarchical SOM (HSOM) and were grouped to neuron areas on the basis of vicinity in the hexagonal output grid. While primary neuron separation and HSOM resulted in classifications on a level more sensitive than species differences, neuron areas resulted in chemical phenotypes apparently of the order of species. These chemical phenotypes have implications for systematics: while the chemical phenotypes for *T. ferox* and *T. moravicum* correspond to morphological determination, in *T. caespitum* and *T. impurum* a total of six chemical phenotypes is found. Three hypotheses are discussed to explain this disparity between morphological and chemical classifications, including in particular the possibility of hybridization and the existence of cryptic species. Overall, the GC-MS profiles classified by SOM prove to be a practical alternative to morphological determination (*T. ferox*, *T. moravicum*) and indicate the need to revisit systematics (*T. caespitum*, *T. impurum*).

Key Words—Self-organizing maps (SOM), *Tetramorium* ants, Formicidae, cuticular hydrocarbons, chemical signature, gas chromatography-mass spectrometry, data mining, systematics, hybridization, cryptic species.

[1] Partly presented as poster (Nikiforov et al., 2001).

* To whom correspondence should be addressed. E-mail: h9304696@edv1.boku.ac.at

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INTRODUCTION

Many aspects of ant behavior such as conspecific and allospecific recognition (e.g., Errard and Vienne, 1994) and chemical mimicry (e.g., D'Ettorre and Errard, 1998) rely on a chemical signature in which hydrocarbons play a major role (Nelson and Blomquist, 1995). In central Europe, five species of *Tetramorium* ants are known: *T. caespitum*, *T. impurum*, *T. rhenanum*, *T. moravicum*, and *T. ferox* (Seifert, 1996). They belong to the *caespitum* group, which was not included in the fundamental worldwide revision of the genus (Bolton, 1980). Since then, the morphology (e.g., Schulz, 1996), genetics (e.g., Baur et al., 1996), and pheromones (e.g., Morgan and Ollett, 1987) of the central European *Tetramorium* species have been repeatedly investigated in systematic context. Nevertheless, the systematic position of these ants remains unclear (Seifert, 1996). Investigations on cuticular hydrocarbons of the central European species have not yet been undertaken. Morphological determination of the known species of this region is difficult and in some cases not unequivocal (e.g., Schlick-Steiner and Steiner, 1999).

Cuticular hydrocarbon profiles are species-specific (e.g., Bagnères and Morgan, 1991) and are, at least partially, due to genetic inheritance (Bonavita-Cougourdan and Clément, 1994, Dahbi et al., 1998). Furthermore, although our knowledge of the various roles played by hydrocarbons has increased steadily (Blomquist et al., 1998), GC-MS data of cuticular hydrocarbons have only rarely been used for systematic purposes in ants (Nowbahari et al., 1990), unlike in termites (e.g., Haverty et al., 1988) and bark beetles (e.g., Page et al., 1997).

So far, studies comparing different cuticular hydrocarbon patterns in social insects have used descriptive linear mathematical techniques (e.g., Haverty et al., 1990, Bonavita-Cougourdan and Clément, 1994) with the exception of Bagnères et al. (1998), who applied multilayer perceptron, i.e., a supervised artificial neural network, for caste separation within termite populations by using different selections of cuticular hydrocarbons as input.

Self-organizing maps (SOM) were introduced in 1981 (Kohonen, 2001) and represent a neural network algorithm based on unsupervised training (Kohonen, 2001). They have an immense advantage over supervised algorithms such as multilayer perceptron since the final target values of any vectors need not be known. Up to now SOM have been successfully used in numerous applications, including image analysis and exploratory data analysis. Therefore, they have become an important tool in data mining, in knowledge discovery in databases (KDD), and especially in assessing classifications.

In this work, we applied SOM based on a hexagonal output grid of 8×6 neurons for comparison and classification of components intensity profiles of GC-MS data of cuticular hydrocarbons from samples of four central European *Tetramorium* ant species. This investigation is part of a multidimensional approach to systematics and determination of the central European species of *Tetramorium*.

METHODS AND MATERIALS

Animals. Workers from a total of 63 colonies of *T. caespitum* ($N = 32$), *T. impurum* ($N = 13$), *T. moravicum* ($N = 13$) and *T. ferox* ($N = 4$) were collected in eastern Austria and Hungary (Table 1) between June and October 2000. One colony of *T. semilaeve* (Sant Cugat, Spain) was added as an outgroup. Ants were collected with an aspirator made of Teflon and glass components, which was frequently cleaned with *n*-heptane p.A., and then transferred to clean glass vials with Teflon-coated caps. After anesthesia by cooling to -21°C, the ants were stored at this temperature until further processing.

Morphological Determination. Nest series were morphologically determined according to the key in Seifert (1996).

Extraction of Cuticular Hydrocarbons. Ants were warmed to room temperature. Five workers from each colony that were ensured not to be callow workers [which have slightly different hydrocarbon profiles (Dahbi et al., 1998)], were extracted in 80 μl of *n*-heptane for 10 min, in order to obtain extracts robust to little variation of extraction time (Vander Meer et al., 1989). While in similar studies pentane (e.g., Nowbahari et al., 1990) or hexane (e.g., Vander Meer and Lofgren, 1985) were used, heptane combines the advantage of a higher boiling point and better quality of GC-MS data. In order to test reproducibility, we additionally extracted four single workers from at least one colony of each species.

GC-MS Analysis. Extracts were analyzed by coupled gas chromatography–mass spectrometry (GC-MS) on a Hewlett-Packard 5973 mass selective detector coupled with a HP 6890 GC-system. MS-spectra were recorded in EI mode at 70 eV, within a mass range of 40–500 mass units and a scan cycle time of 0.7 sec. A detector temperature of 250°C and an injector temperature of 300°C were chosen as GC conditions. A Carbowax column (25 m, 0.25 mm) was used. Temperature was programmed from 100°C to 150°C at 30°C/min and from 150°C to 300°C at 5°C/min. The carrier gas was helium (1 bar). A 1-μl sample was injected in splitless mode.

Profiling of GC-MS Data. For profiling (repeated comparison) of chromatograms, some suitably defined standard format as vector entities is necessary. At the onset, we used components intensity profiles of 71 and 41 selected components, respectively, i.e., a vector of dimensionality of 71 and 41, respectively. The GC-MS profile of a single sample with 71 components was used as primary or master GC after corresponding synchronization (using relative intensity normalization and a combination of "offset" and "stretch") with secondary GC (and in turn with all remaining samples) to detect these 71 peaks in secondary GC (and in turn in all remaining samples) and to determine their peak heights. MS data are not necessary for the generation of the resulting components profiles, but the mass spectra in the GC-MS data provide additional proof that the corresponding peaks are really derived from the same compound.

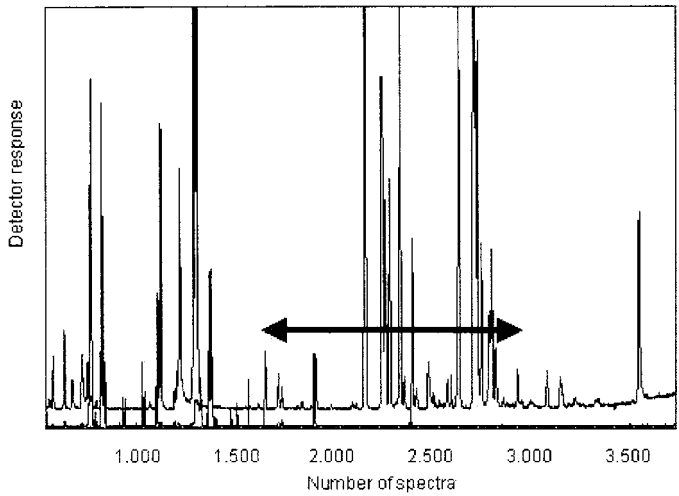


FIG. 1. The 41 hydrocarbon intensity peaks (peaks 26–66, range indicated by arrow) are selected for the final analysis by PCA and SOM; SID (single ion detection) for a fatty acid ethylester (below).

Selection of Hydrocarbons by PCA. To ensure that only hydrocarbons were selected in the data analysis, PCA was performed on mass spectra contributing to a given sample (relative proportions of all compounds $>0.2\%$ of TIC), sorting out fatty acids and all other compounds. Only the remaining 41 hydrocarbon intensity peaks (peaks 26–66 $=$ 41; Figure 1) were used for analysis. While the signature that is detected by nestmates and foreign individuals is an entire mixture of substances on the cuticular surface, our selection of a narrow band represents a compromise to optimally correlate the characteristics of the analytical interpretation with the morphological determination.

Self-Organizing Maps (SOM). SOM visualization was based on a hexagonal output grid of 8×6 neurons. Neurons of the first round (primary neuron separation), which contained at least three samples, were subjected to another round (hierarchical SOM, HSOM) of separation, resulting in subneuronal classification. Hexagonal output grid and u-Matrix were used for visualizing similarities between neurons and subneurons, respectively. According to these, neuron areas were formed for grouping of data on a higher scale.

RESULTS

Classification of GC-MS Data by PCA and SOM. PCA was not able to separate the samples when all components intensity peaks were included in data analysis. SOM led to a grouping of 26 neurons (Table 1). After selection of hydrocarbons

TABLE 1. CLASSIFICATION (PRIMARY NEURON SEPARATION AND HIERARCHICAL SOM) OF 84 SAMPLES BY SOM[a]

Sample	Extracted workers (*N*)	Geographic origin	Month of collection	Morphological determination	Neuron No.		
					71 components	41 components	
					Primary neuron separation	Primary neuron separation	Hierarchical SOM
TM51	5	Sant Cugat (E)	7	*T. semilaeve*	5	1	1-1
TM51a	1	Sant Cugat (E)	7	*T. semilaeve*	5	1	1-8
TM51b	1	Sant Cugat (E)	7	*T. semilaeve*	5	1	1-1
TM51c	1	Sant Cugat (E)	7	*T. semilaeve*	5	1	1-9
TM47	5	Orth/Donau (A)	9	*T. caespitum*	3	3	
TM59	5	Ratzersdorf/St. Pölten (A)	9	*T. caespitum*	3	3	
Blank3					46	5	5-4
TM17a	1	Leopoldsberg (A)	6	*T. caespitum*	6	5	5-17
TM24	5	Hohe Wand (A)	8	*T. impurum*	5	5	5-1
TM42a	1	Hohe Wand (A)	8	*T. impurum*	5	5	5-3
TM42b	1	Hohe Wand (A)	8	*T. impurum*	5	5	5-1
TM42c	1	Hohe Wand (A)	8	*T. impurum*	5	5	5-1
TM42d	1	Hohe Wand (A)	8	*T. impurum*	5	5	5-6
TM44	5	Hohe Wand (A)	8	*T. impurum*	7	5	5-1
TM45	5	Hohe Wand (A)	8	*T. impurum*	7	5	5-1
TM48	5	Hohe Wand (A)	8	*T. impurum*	5	5	5-1
TM49	5	Hohe Wand (A)	8	*T. impurum*	5	5	5-1
TM51d	1	Sant Cugat (E)	7	*T. semilaeve*	5	7	
TM52	5	Setzberg/Wachau (A)	9	*T. impurum*	15	16	16-2
TM54	5	Höflein/Donau (A)	9	*T. impurum*	12	16	16-6
TM60	5	Setzberg/Wachau (A)	9	*T. caespitum*	15	16	16-10
TM80	5	Oberweiden (A)	9	*T. caespitum*	5	16	16-3
TM34a	1	Csiky Hegiek (H)	6	*T. caespitum*	7	18	
TM34c	1	Csiky Hegiek (H)	6	*T. caespitum*	7	18	
TM18	5	Hackelsberg (A)	6	*T. ferox*	6	19	19-1

TABLE 1. CONTINUED

Sample	Extracted workers (*N*)	Geographic origin	Month of collection	Morphological determination	Neuron No.		
					71 components	41 components	
					Primary neuron separation	Primary neuron separation	Hierarchical SOM
TM25	5	Braunsberg (A)	6	*T. ferox*	5	19	19-1
TM8	5	Hundsheimer Berg (A)	6	*T. ferox*	8	19	19-7
TM9	5	Hundsheimer Berg (A)	6	*T. ferox*	18	19	19-13
TM9a	1	Hundsheimer Berg (A)	6	*T. ferox*	18	19	19-13
TM9b	1	Hundsheimer Berg (A)	6	*T. ferox*	18	19	19-15
TM9c	1	Hundsheimer Berg (A)	6	*T. ferox*	12	19	19-15
TM35	5	Wiener Neustadt (A)	9	*T. caespitum*	12	21	21-1
TM55	5	Wiener Neustadt (A)	9	*T. impurum*	12	21	21-3
TM79	5	Setzberg/Wachau (A)	9	*T. caespitum*	14	21	21-8
TM33	5	Oberweiden (A)	9	*T. caespitum*	18	24	
TM63	5	Forchtenstein (A)	9	*T. impurum*	18	24	
TM34	5	Csiky Hegiek (H)	6	*T. caespitum*	1	26	
TM78	5	Oberweiden (A)	9	*T. caespitum*	18	29	
TM37	5	Wiener Neustadt (A)	9	*T. impurum*	30	30	30-1
TM40	5	Perchtoldsdorfer Heide (A)	7	*T. caespitum*	27	30	30-12
TM41	5	Perchtoldsdorfer Heide (A)	7	*T. caespitum*	19	30	30-13
TM46	5	Ratzersdorf/St. Pölten (A)	9	*T. caespitum*	30	30	30-3
TM67	5	Setzberg/Wachau (A)	9	*T. impurum*	30	30	30-11
TM76	5	Forchtenstein (A)	9	*T. caespitum*	30	30	30-1
TM77	5	Ratzersdorf/St. Pölten (A)	9	*T. caespitum*	39	30	30-13
TM84	5	Hüttelberg (A)	10	*T. caespitum*	39	30	30-2
TM22	5	Hackelsberg (A)	6	*T. caespitum*	7	31	31-7
TM36	5	Csiky Hegiek (H)	6	*T. caespitum*	12	31	31-8
TM38	5	Csiky Hegiek (H)	6	*T. caespitum*	7	31	31-2
TM17d	1	Leopoldsberg (A)	6	*T. caespitum*	34	41	41-7
TM39	5	Orth/Donau (A)	9	*T. caespitum*	19	41	41-9
TM53	5	Orth/Donau (A)	9	*T. caespitum*	28	41	41-1

TM81	5	Wien Innenstadt (A)	9	*T. caespitum*	34	41	41-3
TM34b	1	Csiky Hegiek (H)	6	*T. caespitum*	44	42	
TM34d	1	Csiky Hegiek (H)	6	*T. caespitum*	43	42	
TM10	5	Hundsheimer Berg (A)	6	*T. moravicum*	24	43	43-20
TM14	5	Hundsheimer Berg (A)	6	*T. moravicum*	24	43	43-18
TM15	5	Hundsheimer Berg (A)	6	*T. moravicum*	24	43	43-21
TM19	5	Hackelsberg (A)	6	*T. caespitum*	7	43	43-1
TM2	5	Hundsheimer Berg (A)	6	*T. moravicum*	7	43	43-2
TM20	5	Hackelsberg (A)	6	*T. moravicum*	7	43	43-2
TM21	5	Hackelsberg (A)	6	*T. moravicum*	7	43	43-4
TM23	5	Hundsheimer Berg (A)	6	*T. moravicum*	7	43	43-4
TM29	5	Hundsheimer Berg (A)	6	*T. moravicum*	5	43	43-9
TM30	5	Hundsheimer Berg (A)	6	*T. moravicum*	7	43	43-11
TM4	5	Hundsheimer Berg (A)	6	*T. moravicum*	24	43	43-14
TM5	5	Hundsheimer Berg (A)	6	*T. moravicum*	24	43	43-18
TM6	5	Hundsheimer Berg (A)	6	*T. moravicum*	24	43	43-17
TM7b	1	Hundsheimer Berg (A)	6	*T. moravicum*	47	43	43-9
TM7c	1	Hundsheimer Berg (A)	6	*T. moravicum*	48	43	43-1
TM7d	1	Hundsheimer Berg (A)	6	*T. moravicum*	24	43	43-7
TM31	5	Csiky Hegiek (H)	6	*T. caespitum*	7	44	
TM11	5	Praterbahnhof (A)	6	*T. caespitum*	19	45	
TM12	5	Praterbahnhof (A)	6	*T. impurum*	24	46	46-5
TM13	5	Praterbahnhof (A)	6	*T. caespitum*	24	46	46-2
TM83	5	Wien Innenstadt (A)	9	*T. caespitum*	31	46	46-8
TM27	5	Nußberg (A)	8	*T. caespitum*	19	47	47-1
TM32	5	Nußberg (A)	8	*T. caespitum*	19	47	47-9
TM74	5	Oberweiden (A)	9	*T. caespitum*	26	47	47-9
TM75	5	Oberweiden (A)	9	*T. caespitum*	39	47	47-11
TM82	5	Wien Innenstadt (A)	9	*T. caespitum*	34	47	47-12
TM17b	1	Leopoldsberg (A)	6	*T. caespitum*	44	48	48-2
TM17c	1	Leopoldsberg (A)	6	*T. caespitum*	36	48	48-2
TM7a	1	Hundsheimer Berg (A)	6	*T. moravicum*	47	48	48-8
TM9d	1	Hundsheimer Berg (A)	6	*T. ferox*	44	48	48-9

[a] Based on all 71 and on the selected 41 components intensity peaks; morphological determination, geographic origin, month of collection and number of extracted workers.

(reduction of components intensity peaks from 71 to 41) as described above, PCA was still not able to sort the data. SOM resulted in a total of 21 neurons (Table 1, Figure 2) visualized in a hexagonal output grid (Figure 3). HSOM (performed on the 12 primary neurons containing at least three samples) resulted in a total of 39 subneurons (2–5 subneurons per neuron, Table 1).

Correlations between Morphological Determination and GC-MS Profiles. As distance between neurons in the hexagonal output grid (Figure 3) reflects similarity of GC-MS data, neighboring neurons are grouped to neuron areas. Neurons containing only measurements of single workers are not taken into consideration. We define nine neuron areas (Figure 4): four areas, one for each of *T. semilaeve*, *T. moravicum*, *T. ferox*, and *T. caespitum*, and five neuron areas containing *T. caespitum* together with *T. impurum*.

Table 1 lists the neuronal classification of a total of 84 GC-MS profiles (60 profiles of five pooled workers, 24 profiles of single workers), the corresponding morphological determinations, and the sample sites. The four pooled samples of *T. ferox* are classified as belonging to neuron 19, those of *T. moravicum* (13 samples) to neuron 43. Furthermore, *T. semilaeve* from Spain, used as outgroup, was distinct (neuron 1) from the ingroup taxa. In contrast, samples morphologically determined as *T. impurum* are mixed together with some samples of *T. caespitum* in five neurons (neuron 5, 16, 21, 30, 46). The rest of the *T. caespitum* samples are classified into eight separate neurons (neuron 3, 29, 31, 41, 42, 44, 45, 47). Primary neuron separation partially seems to differentiate on a level that is more sensitive than the species level.

The subneuronal classification results in a high number of subneurons per species. The numbers of undivided entities (subneurons plus neurons contain-

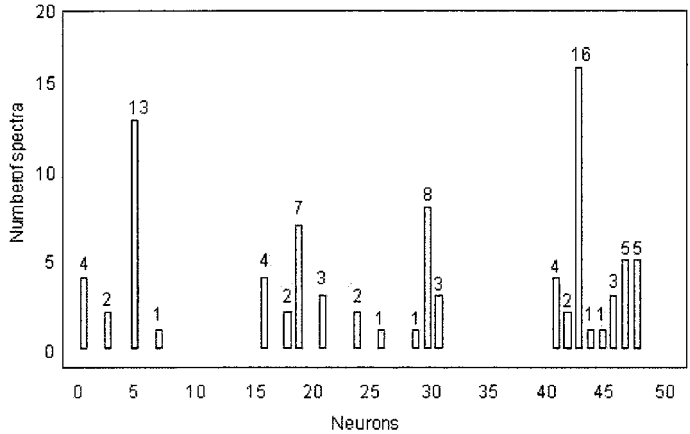


FIG. 2. Results of SOM based on 41 components intensity peaks of the 84 samples, visualized as number of samples per neuron.

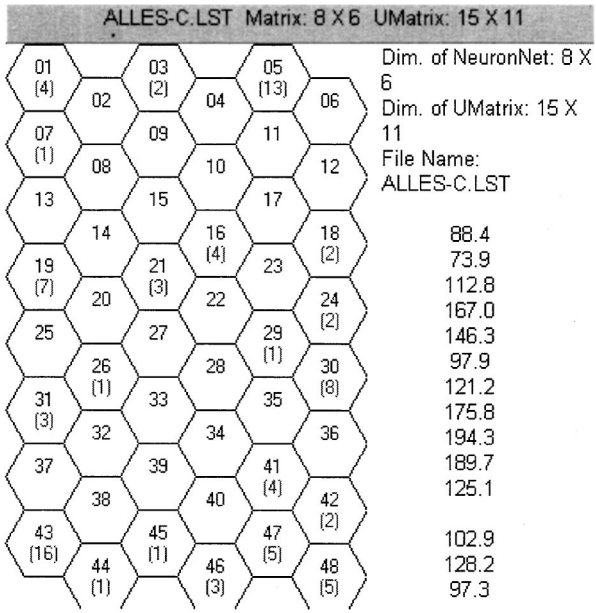


FIG. 3. Results of SOM based on 41 components intensity peaks of the 84 samples, visualized as hexagonal output grid. Numbers in hexagons: neuron number (above), number of samples per neuron (below).

ing less than three profiles, profiles of singular workers excluded) are 29 for *T. caespitum* (32 nests), 8 for *T. impurum* (12 nests), 9 for *T. moravicum* (13 nests), and 3 for *T. ferox* (4 nests), and the total number of undivided entities is 49, as Table 1 reveals. Nevertheless there still is one subneuron containing a profile of each *T. caespitum* and *T. impurum*. Overall HSOM seems to differentiate at a level much more sensitive than between species.

Correlations between Geographic Origin and GC-MS Profiles. There is no correlation between sampling sites and neuronal classification of the GC-MS hydrocarbon profiles. Instead, a case-by-case comparison of two conspecific nests (*T. caespitum*) from sampling sites about 80 km apart shows similar profiles (Figure 5) that are classified as neuron 3, which represents a separate neuron area. SOM analysis of the data set results in similar conclusions (Table 1): Different nests of *T. moravicum* (of which systematic status and morphological determination have never been questioned) from the same sampling site belong to different subneurons (TM20, TM21), while nests of *T. moravicum* from two sampling sites, 22 km apart, belong to the same subneuron (TM21, TM23).

Correlation between Date of Collection and GC-MS Profiles. According to the SOM analysis (Table 1), there is no evident correlation between the neuronal

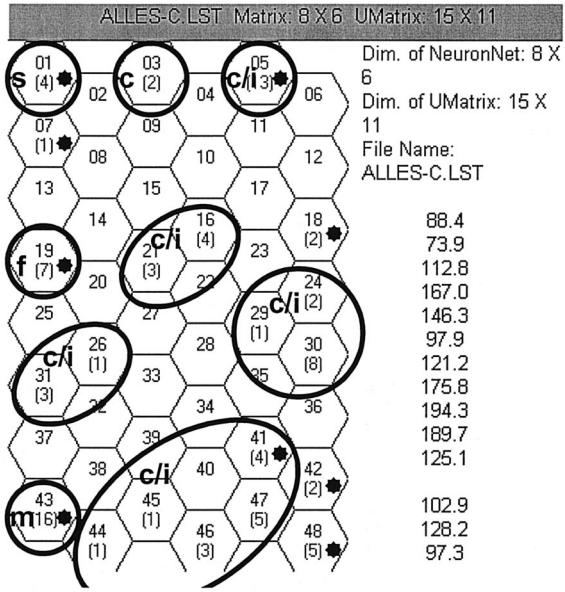


FIG. 4. Nine neuron areas are defined, based on the location of the neurons in the hexagonal output grid. Neurons containing exclusively single workers are not taken into consideration. (Numbers in hexagons: neuron number above, number of samples per neuron below, neurons containing samples of single workers marked with asterisks, c = *T. caespitum*, c/i. = *T. caespitum* and *impurum*, f = *T. ferox*, m = *T. moravicum*, s = *T. semilaeve*).

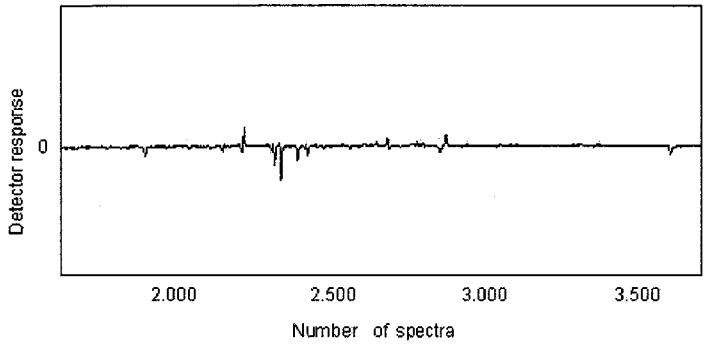


FIG. 5. Resulting residual intensities after subtraction of the two GC-MS components intensity profiles of TM47 and TM59.

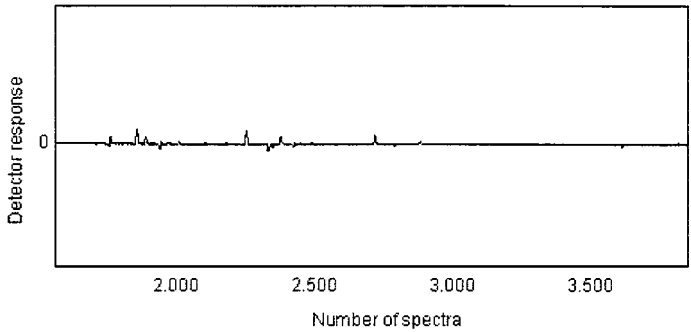


FIG. 6. Resulting residual intensities after subtraction of the two GC-MS components intensity profiles of TM42b and TM42c.

classification of the GC-MS hydrocarbon profiles and the date of collection. Different nests of *T. caespitum* sampled in the same location and on the same date (July 2) belong to different subneurons (TM40, TM41), while nests of *T. caespitum* collected in July and September belong to the same subneuron (TM41, TM47).

DISCUSSION

Reproducibility. For all species, four individual workers from the same nest were separately tested for the reproducibility of GC-MS measurements and similarity of profiles. The profiles of two *T. impurum* workers from the same nest are similar. After subtraction of one profile from the other, only weak residual intensities remain (Figure 6). However, this is not true in all cases: one of four single measurements of *T. ferox* and *T. moravicum* was classified as belonging to a different neuron. This result is surprising since the similarity of profiles of individual workers from the same nest has been proven repeatedly (e.g., Nowbahari et al., 1990). It is known that quantitative intracolonial variations may be due to different categories of workers (Bonavita-Cougourdan and Clément, 1994) and may be influenced by endoparasites (Trabalon et al., 2000). Yet, these factors cannot explain all the variation in our data set, as in some cases the variation exceeds the level of interspecific variation. One explanation for this may be related to the technical problem of measuring the level of chemicals in single workers due to a low intensity of component peaks. Measurements of single individuals are technically possible, but since they are accompanied by a reduced reliability, further consideration will be restricted to the pooled measurements of five workers only.

Comparison of PCA and SOM for Classification of Hydrocarbon Patterns. The results obtained by PCA and SOM reveal the greater ability of SOM to separate the data. PCA uses a simple projection of data points on a plane defined mostly

by the first and second eigenvectors, whereas SOM uses so-called weight vectors that are placed within and near the data vectors after the training epochs. The nearby locations in the input space correspond to the neighboring neurons. These weight vectors define centers of so-called Voronoi regions (Kohonen, 2001). The data within these are assigned to the particular weight vector and visualized by the particular net neuron; thus, the number of weight vectors equals the number of neurons. In the final state, the weight vectors are laid down like a flexible net over the hypersurface built by the data vectors. Thus, SOM reveals much more information about the structure of the data than PCA. In contrast, PCA is always totally reproducible, whereas visualization of SOM results is dependent in part on: (1) the structure of the SOM used, (2) the order of data input (because it is practically impossible to choose totally isotropic means of visualization and, therefore, the position of the neurons in the chosen means of visualization is slightly distorted), and (3) the number of training epochs.

Correlations between Geographic Origin and GC-MS Profiles. Dahbi et al. (1996) found marked quantitative differences between separate populations (600 km apart) of the ant *Cataglyphis iberica*. No population genetic data concerning the central European species of *Tetramorium* are available to decide whether the investigated nests of eastern Austria and Hungary belong to separate populations. Nonetheless, the distance between the sample sites of eastern Austria (maximum distances of 105 km) and the lack of geographic separation suggest that they belong to the same population. Whether the more distant nests of *T. caespitum* from Hungary (180 km to the next *T. caespitum* sample site in Austria) belong to a separate population cannot be decided on the basis of our data set. Since the differences of GC-MS data are of the same order of magnitude as those between the Austrian nests of *T. caespitum*, we think the differences found are not due to geographic variation but to other reasons. We conclude that all samples of each of the four species sampled in central Europe belong to a continuous population.

Correlation between Time of Collection and GC-MS Profiles. Quantitative variations between samples of one nest over a period of several months have been reported (Vander Meer et al., 1989; Nielsen et al., 1999). These investigations were designed to detect potential season specificity of hydrocarbon patterns in one species and, thus, it was important to collect samples at one particular date (Nielsen et al., 1999). We chose to collect samples over a five-month period to take into account possible seasonal variations and to detect differences among the *Tetramorium* nest profiles that may be due to phylogenetic distance. Because of this sampling strategy, significant differences in the hydrocarbon profiles should be more robust and stable to seasonal variations.

Chemical Phenotypes and Implications for Systematics. The GC-MS profiles of the samples in the nine neuron areas, which we defined (Figure 4), are distinct enough to be considered as chemical phenotypes. The morphologically determined *T. moravicum*, *T. ferox*, and *T. semilaeve* coincide with distinct chemical

phenotypes. However, this is not the case for *T. caespitum* and *T. impurum*, where six distinct chemical phenotypes occur that are characterized by quantitative and qualitative differences. So far, only quantitative variations among conspecific workers of different colonies are known (Morel et al., 1988, Yamaoka, 1990, Bonavita-Cougourdan and Clément, 1994, Nielsen et al., 1999). Our results contradict Billen et al. (1986), who found that contents of Dufour glands of workers of *T. caespitum* and *T. impurum* from England, Belgium, and France are similar, but species-specific. Our results support the findings of Schlick-Steiner and Steiner (1999), where morphological determination of nest series of *T. caespitum* and *T. impurum* from Vienna was not unequivocally possible in all cases. Although only two ant species are concerned, the SOM analysis reports the occurrence of six chemical phenotypes. Biological explanations for this are discussed in the following three hypotheses.

1. The theory of a species-specific cuticular hydrocarbon profile could not be demonstrated for these species. This hypothesis is unlikely and does not hold true for the central European species *T. moravicum* and *T. ferox*, which partially occur syntopically with *T. caespitum* and *T. impurum*. In fact, it would contradict all published ethological and sociobiological findings in this field (see references in the introduction).

2. A natural hybridization of *T. caespitum* and *T. impurum* occurs. This phenomenon has been shown to exist in ants by chemical, genetic, and morphological means [e.g., *Solenopsis invicta* and *S. richteri* (Vander Meer and Lofgren, 1985; Diffie et al., 1988; Ross et al., 1987), *Leptothorax nigriceps* and *L. unifasciatus*, (Seifert, 1984)]. In the case of *S. invicta* and *S. richteri*, it was more evident from the GC-MS data of the cuticular hydrocarbons, since a clear intermediate chemical phenotype existed that combined components of both species. However, this does not necessarily occur with hybrids, especially when the hybrids are fertile in two or more generations, as discovered by Buschinger (2001) when back-crossing occurs and when the chemical signature is expressed in a dominant–recessive mode. If this hypothesis is true, *T. caespitum* and *T. impurum* would remain as valid systematic entities. It can be expected that morphological characters for the determination of hybrids will be found, as they are known for hybrids of other ant species [e.g., *Formica rufa* x *polyctena* (Seifert, 1996)].

3. One or more cryptic species exists. Morphological analyses of central European ants have repeatedly split up frequently occurring species into one or more species [*Lasius niger* and *L. platythorax* (Seifert, 1991); *Lasius alienus*, *L. paralienus*, and *L. psammophilus* (Seifert, 1992)]. In addition, the application of methods from other disciplines further facilitates our knowledge of systematics at this high level [Haverty et al. (1988) for termites, Pinto et al. (1997) for Trichogrammatidae, Nowbahari et al. (1990) for the ant *Cataglyphis cursor*]. If this hypothesis is true, it would entail many changes in systematics and determination.

Hence, while recognizing the need for other analyses, especially on morphology and genetics, to evaluate these hypotheses and, in general, to elucidate the condition of *Tetramorium* spp. in central Europe, the results of the presented SOM classification will be used as a basis on which to map further results. The rather intuitive approach of SOM (cf., Kohonen, 2001) facilitates positing hypotheses, which by further approaches can be verified, modified, or completely falsified. In a similar way, classification by SOM can be readily applied to other chemical, ecological, and systematic studies of social insects, in particular where prior work on clearly identifiable groupings is absent. Furthermore it could be tested in neighboring fields such as population genetics.

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SEMIOCHEMICALS RELEASED BY ELECTRICALLY STIMULATED RED IMPORTED FIRE ANTS, *Solenopsis invicta*

R. K. VANDER MEER,[1,*] T. J. SLOWIK,[2,3] and H. G. THORVILSON[2]

[1]*United States Department of Agriculture—Agricultural Research Service*
Center for Medical, Agricultural and Veterinary Entomology
1600 SW 23rd Dr., Gainesville, Florida 32608, USA

[2]*Texas Tech University*
Lubbock, Texas 79409-2l22, USA

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Abstract—The red imported fire ant *Solenopsis invicta* Buren, has evolved sophisticated chemical communication systems that regulate the activities of the colony. Among these are recruitment pheromones that effectively attract and stimulate workers to follow a trail to food or alternative nesting sites. Alarm pheromones alert, activate, and attract workers to intruders or other disturbances. The attraction and accumulation of fire ant workers in electrical equipment may be explained by their release of pheromones that draw additional worker ants into the electrical contacts. We used chemical analysis and behavioral bioassays to investigate if semiochemicals were released by electrically shocked fire ants. Workers were subjected to a 120 V, alternating-current power source. In all cases, electrically stimulated workers released venom alkaloids as revealed by gas chromatography. We also demonstrated the release of alarm pheromones and recruitment pheromones that elicited attraction and orientation. Arrestant behavior was observed with the workers not electrically stimulated but near those that were, indicating release of unkown behavior-modifying substances from the electrically stimulated ants. It appears that fire ants respond to electrical stimulus by generally releasing exocrine gland products. The behaviors associated with these products support the hypothesis that the accumulation of fire ants in electrical equipment is the result of a foraging worker finding and closing electrical contacts, then releasing exocrine gland products that attract other workers to the site, who in turn are electrically stimulated.

Key Words—Semiochemicals, pheromones, alarm, attraction, electric field, fire ant, behavior, recruitment.

[3] Current address: Insect Biology Division, ESPM Department, University of California—Berkeley, Berkeley, California 94720-3112, USA.
[*] To whom correspondence should be addressed. E-mail bobvm@ufl.edu

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INTRODUCTION

The red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), occurs in densities at least five times greater in the southern and southeastern United States than in its native South American habitat (Porter and Savignano, 1990; Porter et al., 1992). This large biomass of aggressive fire ants dominates the ecological community (Porter and Savignano, 1990) and interferes with human activities (Lofgren, 1986). One unusual effect on human activities is fire ant damage to electrical equipment, in which large numbers of ants invade outdoor electrical apparatuses, create short-circuits, foul conductive materials, and jam internal mechanisms (Eagleson, 1940; Vinson and MacKay, 1990), all of which can lead to destruction of circuitry.

Alternating-current frequencies, heat, ozone, magnetic fields, and wire insulation have no effect on ants (MacKay et al., 1989). Fire ants were initially reported to be attracted to electrical fields along with several other ant species (MacKay et al., 1992a,b). However, Slowik et al. (1996) reported that fire ants are not attracted to electrical fields, but instead probably release chemicals that excite and attract other ants to the release site after ants contact bare, bridgeable conductive surfaces, such as exposed wires or contact points.

Electrical stimulation of worker ants causes incapacitation, death, and aggression towards sister workers (Slowik et al., 1996). In addition, both voluntary (not induced by electrical stimulation) and involuntary (displayed by electrically stimulated ants) "gaster-flagging" was observed (Obin and Vander Meer, 1985; Slowik et al., 1996). Gaster flagging signals the release of defensive venom alkaloids (Obin and Vander Meer, 1985). Therefore, the release of semiochemicals by electrically shocked worker ants in the vicinity of infested circuitry may cause destructive aggregations of fire ants in electrical equipment (Slowik et al., 1996).

Pheromonal chemical cues are the most important form of communication among ants (Vander Meer and Alonso, 1998), and several pheromone systems of *S. invicta* have been investigated. For example, an alarm/excitant pheromone is involved in mating flight activity (Obin and Vander Meer, 1994; Alonso and Vander Meer, 1997). A recruitment pheromone is produced by the Dufour's gland and is released via the sting apparatus (Wilson, 1959). This complex recruitment pheromone system has several concentration- and context-related functions. Pheromones attract workers (Vander Meer et al., 1988), modulate their general responsiveness [i.e., orientation induction (Vander Meer et al., 1990)], and orient workers along a pheromone trail to food (Vander Meer et al., 1981). Fire ant venom is primarily composed of piperidine alkaloids, readily analyzed and well characterized (MacConnell et al., 1971; Leclercq et al., 1994). Venom functions in defense against intruders, in procurement of prey, and as an antimicrobial agent to maintain hygienic conditions within a colony (Obin and Vander Meer, 1985).

Venoms of other social hymenopterans such as bees, wasps, and hornets can be experimentally collected by electrical stimulation (O'Connor et al., 1963). In the field, honeybees (*Apis mellifera* L.) housed near extra-high-voltage power lines are induced to sting and release venom when electrified by as little as 900 nA of current after alighting on wet hive entrances (Bindokas et al., 1988). Fire ants, like honeybees, may also release venom and perhaps other semiochemicals when electrically shocked. These released chemicals might contribute to the observation that large masses of fire ants are often found in electrical circuitry, causing the equipment to malfunction (Vinson and MacKay, 1990). We report here the results of experiments that investigate, through behavior-specific bioassays and chemical analyses, what pheromones or semiochemicals are released by electrically stimulated fire ants.

METHODS AND MATERIALS

Source of Monogyne Solenopsis invicta Colonies. Queenright monogyne *S. invicta* colonies were obtained by rearing colonies from newly mated queens or by excavation of field nests. Monogyne colonies from the field were characterized by low mound density, well-developed large nests, polymorphic characteristics of colony workers (Greenberg et al., 1985), no more than one queen found, and a high level of intraspecific aggression (Morel et al., 1990). Excavated field colonies were brought to the laboratory only if the colony queen was found, and then the colony workers and brood were isolated by a standard float-out procedure (Banks et al., 1981). All colonies isolated after excavation were placed in plastic rearing trays (52 × 39 × 7.5 cm). Inside walls of trays were painted with Fluon (Dupont, Wilmington, Delaware, USA) to keep ants from escaping. Each colony was provided with Petri dish nest cells (14 cm diam. × 2.5 cm deep) with a Castone substrate (Dentsply Co., York, Pennsylvania, USA) and cotton-stoppered water tubes (Obin, 1986). Colonies were reared on crickets and 10% sugar water absorbed in wads of tissue. Rearing temperatures were maintained between 21 and 28°C, and the light–dark cycle was variable.

Preparation of Electrical Wire. A standard receptacle plug was attached to one end of common electrical cord (ca. 2 m). At the other end of the cord, ca. 2 cm of insulation was removed. Separately, insulation was stripped from 18-gauge wire, except for 5 mm on one end. The 18-gauge wire was soldered to the available end of the common electrical wire. Heat-shrink Teflon tubing was placed over each of soldered connections and wrapped with electrical tape. A piece of heat-shrink Teflon tubing was also placed over the wire ends that still had a small amount of insulation remaining. This functioned to hold the wires ca. 1 mm apart. The bare wires (5 cm long) were bent appropriately to better contact the floor and side of the test vial (20-ml scintillation vials), taking care to keep them 1 mm apart and

not touching each other. Wires were energized by plugging the electrical cord plug into an adjustable power supply containing a 1-A fuse that was connected to a standard 120 V, alternating-current power source.

General Experimental Procedure. Test worker ants (ca. 50) were randomly selected from laboratory-maintained monogyne *S. invicta* colonies (see above) and placed into 20-ml scintillation vials that had their inside lip painted with Fluon to help keep the ants inside the vial. The vial was clamped to a ring stand for stability. The wire was plugged into the adjustable power supply and gently inserted into the vial of ants. Ants were allowed to settle down without electrical stimulation for at least 15 min. The power was turned on and adjusted to 110 V. Ants walking across both bare wires would complete the electrical circuit and become electrically stimulated. After 2 min, the power was turned off and the ants were removed from the wire and vial with feather forceps. The bent end of the wire was placed on the lip of a clean and labeled 20-ml scintillation vial. Hexane (Burdick and Jackson, Muskegon, Michigan, USA; GC^2 grade) was added to the top of the bare wire one drop at a time with a disposable Pasteur pipet and allowed to run down the length of the wire to the bend where it dropped into the vial. This was repeated several times to ensure that all potential compounds released by the ants were removed from the wire. The hexane wire rinse was transferred to an autosampler vial for analysis by GC or for bioassay. The vial in which the worker ants were electrically stimulated was also carefully rinsed with hexane and subjected to chemical analysis or bioassay. If headspace samples (air in the vial) were needed, the vial in which the ants were electrically stimulated was immediately capped after the ants were removed. The procedure for the alarm bioassay is described in detail below.

Analysis of Venom Alkaloids and Cuticular Hydrocarbons. Immediately after an electrical stimulation treatment or control was complete and the worker ants were removed, 10 μl of a 100 ng/μl hexane solution of *n*-tetracosane were added to the test vial as an internal standard. Similarly, prior to rinsing the treatment or control electrical wire with hexane, 10 μl of a 100 ng/μl hexane solution of *n*-tetracosane were added to the collection vial as an internal standard. After addition of the internal standard, the treatment or control samples were treated as described above. Prior to GC analysis, all samples were brought to a volume of 100 μl and transferred to 300-μl autosample inserts.

Gas chromatography (GC) was carried out with a Varian 3700 (Varian, Palo Alto, California, USA) gas chromatograph equipped with a flame ionization detector and a split/splitless injector operated splitless for 0.33 min after which the splitter was turned on (90:1 ratio). Injector and detector temperatures were 300°C. Samples were injected into the system using an autosampler (Leap Technologies, Chapel Hill, North Carolina, USA). Analyses were performed on a DB-1 fused silica column (0.32 mm ID $\times$ 30 m; 0.25-μm film thickness; J & W Scientific, Folsom, California, USA). The oven was programmed from 150 to 285°C at 10°C/min with a 2-min hold. Data were analyzed with Turbochrome Workstation

Version 6.1.0.1 (Perkin Elmer, Norwalk, Connecticut, USA). Peak assignments for the venom alkaloids and cuticular hydrocarbons were based on the GC analysis of authentic *S. invicta* alkaloids and hydrocarbons obtained by overnight soaking of *S. invicta* workers in hexane (see Vander Meer et al., 1985; Ross et al., 1987).

Preparation of Poison and Dufour's Gland Extracts. The poison gland and the Dufour's gland are attached to the sting apparatus. The poison gland produces the piperidine alkaloids that are used by workers in defense and prey procurement. The Dufour's gland is located at the base of the sting and its products exit to the outside via the sting. The Dufour's gland is the source of the fire ant recruitment pheromone. The worker sting apparatus was removed in water with the aid of a dissecting microscope (Leica MZ-8, Heerbrugg, Switzerland) by placing and holding the ant in the water ventral side up. With another pair of forceps, the sting apparatus was removed by grabbing the last segment of the abdomen and pulling it out along with the sting apparatus. Once removed, the fat bodies were taken away, exposing the two glands. At this point, either gland could be obtained by pinching it off where it attaches to the sting apparatus. Excess water was removed by touching the gland carefully to a piece of tissue. The gland was transferred to a probe, then into a vial containing hexane where it was macerated. The volume of hexane was adjusted to give the required concentration in terms of gland equivalents.

Alarm Bioassay. Worker groups were taken from each of 15 mature monogyne *S. invicta* colonies maintained in the laboratory. Approximately 100 workers and a small amount of brood from each colony were placed in small plastic tray ($7 \times 22 \times 5$ cm), whose inner sides were painted with Fluon to prevent escape. The bottom of one half of the tray was covered with a thin layer of moist Castone. Red cellophane was placed over the Castone end of the tray to induce the workers to settle down with their brood. After the ants settled down (at least 30 min), the red cellophane was carefully removed. Tests were carried out only on subcolonies that were calm.

The bioassay required an observer and an assistant. The assistant prepared test samples for the observer so that the observer did not know the sample identity. Test samples consisted of 3 ml of air or headspace (vapor above a sample contained in a vial) drawn into a syringe (5 ml, plastic, Henke-Sass Wolf Co., Tuttlingen, Germany). The assistant randomly assigned each sample to a worker group, so that all worker groups were tested with each of the test samples and controls. The observer positioned the syringe 1–2 cm directly above a selected quiescent worker group, then 1 ml of vapor was slowly released over the workers. Prior to the actual test, the observer determined the blank air release rate that did not initiate a reaction from the worker ants. Reactions were rated on a scale of 1–4 (1 = no reaction, 2 = no more than raised heads and/or antennation of the air, 3 = one worker reacted with rapid movement, and 4 = several workers exhibiting an excited reaction). All tests were conducted with a room temperature of 28°C and standard overhead fluorescent lighting.

Air from an empty vial was tested as a negative control, and 10–25 workers (shaken) were used as a positive control. Shaking disturbed the workers and induced them to release alarm pheromone. Only those series of tests in which appropriate reactions to the negative and positive controls were obtained were used for analysis. Each replicate from a treatment was assayed at least five times, as were the negative and positive controls. We found that greater consistency was obtained for the positive control if there was a vial of workers for each replicate. The following treatment samples were evaluated: Electrically stimulated ants (stimulation time 120 sec); electrically stimulated ants (20 sec); shaken ants (with vial lid off for 120 sec); shaken ants (vial lid off $<$10 sec); and poison sac contents (1 gland equivalent/10 μl hexane absorbed onto a filter paper strip, solvent allowed to evaporate and placed in the bottom of a bioassay vial, headspace taken immediately).

The McNemar test for significance of changes (Sokal and Rohlf, 1981) was used for analysis of the alarm reaction bioassay data. For this analysis, scores of 1–2 were considered no reaction, and 3–4 were counted as alarm. The number of worker groups that displayed an alarm reaction to the test sample but not to the negative control was compared to the number of worker groups that reacted to the negative control but not to the test sample.

Orientation Bioassay. The orientation bioassay presumably measures the ability of workers to detect and follow a chemical trail. The procedure was a modification of that described by Barlin et al. (1976) and Jouvnenaz et al. (1978). Queenright, monogyne colonies were used in the bioassay. The orientation bioassay apparatus consisted of a ramp leading to a test platform (7.5 $\times$ 10 cm), which was connected by a tongue depressor to a similar size platform on which were food items, e.g., crickets or a cotton ball saturated with honey–water (1:1). The legs of the two platforms were painted with Fluon, which forced the ants to move up the ramp and across the test platform to the food platform. A piece of plain paper (7.5 $\times$ 10 cm) was placed on the test platform. The above-described apparatus was set up on a colony tray floor, and worker ants were given time to develop a natural trail to the food platform. Test pieces of paper were prepared by lightly marking in pencil two arcs on opposite sides of the paper from the center of one end to the center of the other end. Test solutions (10 μl) were streaked evenly along one of the arcs, and the appropriate solvent control streaked along the other arc. The test paper was air-dried for about 20 sec, then the natural trail paper on the test platform was replaced with the test paper. If, within 3 min, one ant followed the treatment arc from start to finish, the test was considered positive. A negative test was characterized by confusion at the two trail ends. Samples evaluated were the wire rinse (A) and the vial rinse (B) from electrically stimulated workers in the vial, the wire rinse (C) and vial rinse (D) from nonelectrically stimulated workers in a vial, and (E) the hexane vial rinse of worker ants in a vial without the wire. There were five replicate samples for each of the treatments. All samples

from electrically stimulated ants were adjusted to 250 μl total volume of hexane prior to use. Samples that did not give a positive orientation bioassay were concentrated to 50 μl volume and retested in the bioassay. If the samples did not show activity at this concentration, they were assumed to not have measurable activity.

The Dufour's Gland Equivalents Released by Electrically Stimulated Ants. Samples that showed positive orientation bioassay activity were systematically diluted with hexane until the samples were inactive. Dufour's gland extracts were prepared at a starting concentration of 1 Dufour's gland equivalent (DE) per 10 μl. Serial (1:2) dilutions were made with hexane, and orientation bioassays conducted until the extract was inactive. This was repeated three times. The Dufour's gland equivalent deposited by electrically stimulated ants on the wire and in the vial was determined by comparing the last active concentration of Dufour's gland extracts with the last active dilution of the wire and vial samples from electrically stimulated ants. Knowing the starting volume and the dilution regime allowed the estimation of Dufour's gland equivalents in the wire and vial samples.

Olfactometer Bioassay. A Y-tube olfactometer was used to determine if attractant compounds were released by electrically stimulated worker ants. The bioassay apparatus was similar to that described by Vander Meer et al. (1988). The positive standard was 0.3 queen equivalents of dissected and extracted queen poison sacs (poison sacs from 10 polygyne queens in 300 μl of hexane), the source of the queen attractant pheromone. A null hypothesis of equal numbers of ants in each arm was tested with chi-square analysis. Samples collected from electrically stimulated worker ants were evaluated in the olfactometer bioassay only if a positive response was obtained in the orientation bioassay, since workers are 200–300 times more sensitive to recruitment pheromone in the orientation bioassay than in the olfactometer bioassay (Vander Meer, 1986).

RESULTS

The Ant Response. Worker ants calmly walked on the vial surfaces prior to energizing the bare copper wires that were in the test vials. On energizing the system, worker ants bridging the two wires responded by gaster flagging, curling their gaster toward the wire in a stinging movement, and rapid, random movement in the vial. Other workers that were not initially electrically stimulated, reacted with alarm behaviors, including gaster flagging, and moved excitedly toward the wires, where if they made contact with both wires, they too were induced to exhibit the defensive behaviors described above. Interestingly, before the end of the bioassay (2 min), worker ants not on the wire aggregated quietly away from the wire ends. This was in sharp contrast to their rapid movement at the beginning of the test. Approximately 15–25 workers were electrically stimulated during the 2-min course of the experiment (mean $\pm$ SE: 22.8 $\pm$ 1.26 workers, $N = 8$). Some

of these workers did not survive the treatment. The observed reaction of the ants to electrical stimulation suggested that we could expect to find venom alkaloids and evidence for the release of alarm pheromones.

Venom Alkaloids and Cuticular Hydrocarbons. Both classes of compound can be readily analyzed by GC in a single chromatogram (Ross et al., 1987). All expected piperidine alkaloid components (six) characteristic of *S. invicta* were found. The quantitative results for the venom alkaloids are shown in Figure 1. The total amount of venom alkaloid from the wire rinse of electrically stimulated ants was greater than the treatment vial rinse (mean ± SE: 3.33 ± 0.56 μg vs. 0.23 ± 0.04 μg; $N = 5$ and 4, respectively; unpaired t test, $df = 7$; $t = 4.905$, $P = 0.002$) and by inference any of the controls (see Figure 1). This is a direct reflection of the observed worker defensive behaviors induced by electrical stimulation. The wire was the main target of the ants, but gaster flagging and venom-throwing may have also distributed venom onto the inner walls of the vial. In addition, some worker ants not on the wire may have been stimulated to release venom into the vial. The amount of venom found in the treatment vials also was greater than any of the control samples (treatment vial rinse vs. control wire rinse: mean ± SE: 0.23 ± 0.04 μg vs. 0.06 ± 0.01 μg; unpaired t test, $df = 7$, $t = 5.939$, $P = 0.002$). The amount of alkaloid found on the control wire and in the control vial were not

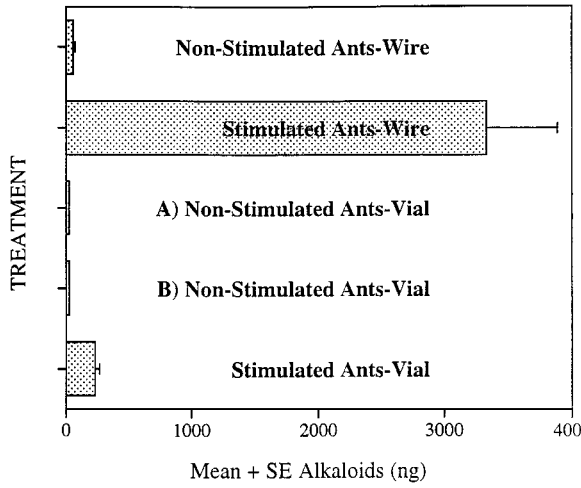


FIG. 1. Results are shown for the quantitative analysis of *S. invicta* venom alkaloids found on the wire or the vial for the control and treatment samples indicated. (A) "Non-stimulated ants-Vial" is the vial rinse that matches the "Non-Stimulated Ants-Wire" sample. This control had the wire inserted as in the electrically stimulated samples but the wire was not energized. (B) "Non-stimulated Ants-Vial" was a control that had worker ants, but did not have the wire inserted. $N = 5$.

different (unpaired t test, $df = 7$, $t = 2.127$, $P = 0.066$). Perhaps the disturbance of putting the wires into the vials with ants induced some of the workers to attack and release some venom on the wire. The mean amounts of venom in control vials were the same regardless of whether the wires were inserted or not (mean $\pm$ SE: 0.03 ± 0.01 μg vs. 0.03 ± 0.01 μg, respectively).

The principal source of cuticular hydrocarbons are from the cuticle of the insect; however, in most ants, including the fire ant, a cephalic lumen, called the postpharyngeal gland, is a copious source of the same hydrocarbon components that are found on the cuticle (Soroker et al., 1994). In our electrical stimulation experiments, the hydrocarbons could be passively transferred from worker cuticle to the wire and/or vial or they could be regurgitated from the postpharyngeal gland through the pharynx and mouth to the wire or vial substrate. Treatment and control vials did not differ in the total amount of cuticular hydrocarbons deposited in the vial during the 15-min acclimation time and the 2-min treatment time (mean $\pm$ SE: 77.0 ± 18.3 ng vs. 78.5 ± 10.4 ng; unpaired t test, $df = 7$, $t = -1.451$, $P = 0.944$).

Alarm Bioassay. Figure 2 shows the results of initial bioassays aimed at determining if electrically stimulated ants released alarm pheromone. Headspace

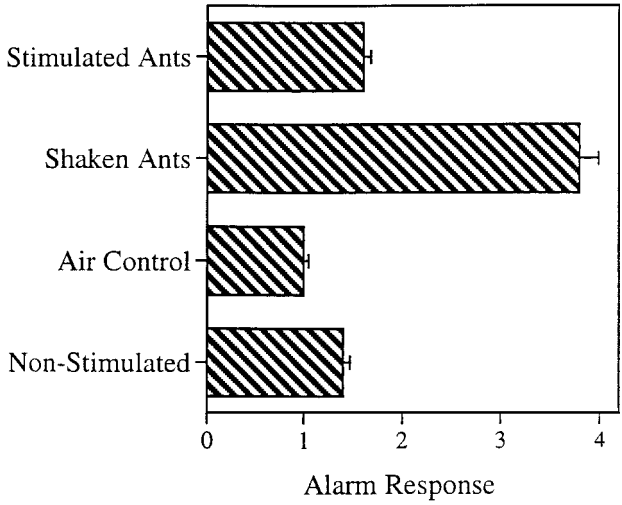


FIG. 2. Alarm bioassay results for headspace from vials in which worker fire ants were electrically stimulated and three controls. Shaken ants represents a positive control. Non-stimulated worker samples are a negative control, and laboratory air is another negative control. The results show that under standard experimental conditions the headspace above electrically stimulated ants does not elicit an alarm response in the alarm bioassay. Results are shown as the mean and SE of 10 replicates.

samples from electrically stimulated ants did not elicit a significantly different alarm reaction from workers than the nonelectrically stimulated controls (McNemar test: $G = 1.04$, $P > 0.05$; $N = 25$), but was different from the shaken ant positive control (McNemar test: $G = 18.5$, $P < 0.001$; $N = 25$). These results did not correspond to expectations based on behavioral observations.

Figure 3 shows the results of shaking worker ants in the vial (positive control) and then not sampling the opened vial for 2 min, the normal duration of the electrical stimulation. Allowing the volatiles generated from the shaken ants to dissipate for 2 min gave a result different from the positive control of shaken ants with the headspace sample taken immediately (McNemar test: $G = 13.86$, $P < 0.001$; $N = 10$). The results for shaken ant samples allowed to stand for 2 min was no different from the unshaken ant negative control (McNemar test: $G = 0$; $P > 0.05$; $N = 10$). Based on this result, we modified our electrical stimulation time to 20 sec, immediately after which the wire was removed and headspace samples were withdrawn for evaluation in the alarm bioassay. The results are shown in Figure 4. The electrically stimulated (20 sec) headspace samples elicited greater alarm response than the appropriate nonstimulated control (McNemar test: $G = 13.86$, $P < 0.001$; $N = 10$; mean $\pm$ SE: 3.9 ± 0.10 vs. 1.4 ± 0.16, respectively). However, there were no differences between the headspace samples of electrically

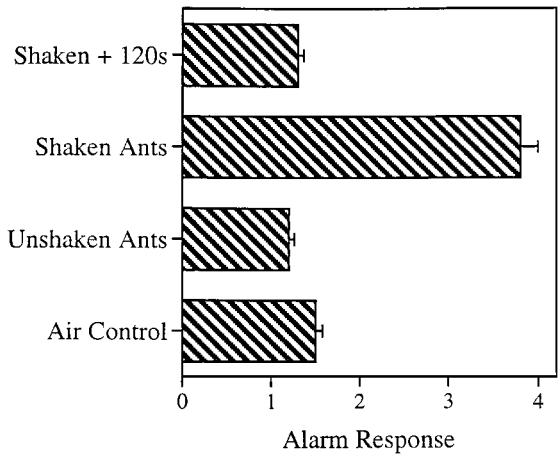


FIG. 3. Alarm bioassay results for headspace from vials in which worker fire ants were shaken to release alarm pheromone. For shaken ants (positive control) the headspace sample was taken immediately after shaking. Samples represented by shaken + 120 sec were shaken as above but the vial was left open for 120 sec prior to taking the headspace sample. Unshaken worker samples were a negative control as was laboratory air. The results show that while shaken ants release alarm pheromone, the pheromone components are highly volatile and evaporate by 120 sec after shaking. Results are shown as the mean and SE of 10 replicates.

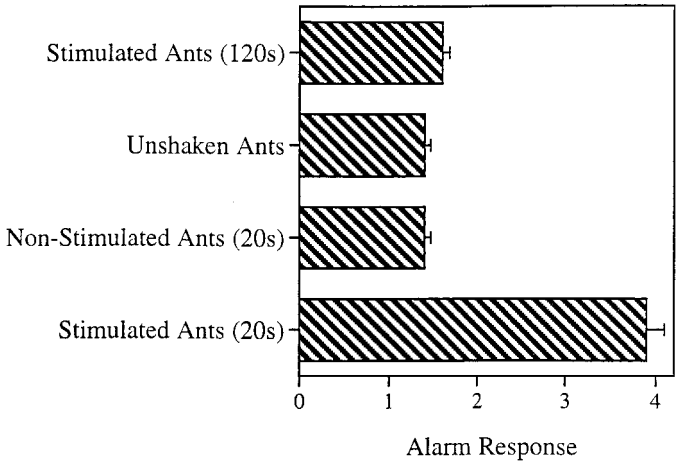


FIG. 4. Alarm bioassay results for headspace from vials in which worker fire ants were electrically stimulated for 120 sec and 20 sec. Unshaken ants was a negative control. Non-stimulated ants (20 sec) was another control where the nonenergized wire was inserted into the vial with worker ants and the headspace sample was taken after 20 sec. The results demonstrate that the alarm pheromone is released early during the electrical stimulation and evaporates out of the vial by the end of the standard 120-sec electrical stimulation time period. Results are shown as the mean and SE of 10 replicates.

stimulated workers and the headspace from the positive control, shaken workers (McNemar test: $G = 1.39$, $P > 0.05$; $N = 10$).

To test whether or not the venom alkaloids were responsible for the observed alarm activity, we applied one poison gland equivalent to a piece of filter paper, allowed the hexane to evaporate, and placed the paper into a typical bioassay vial (20 ml). The vial was capped and the headspace associated with that vial was tested for alarm activity. There was no difference between the air control and the headspace from above the poison sac samples (McNemar test: $G = 1.39$, $P > 0.05$; $N = 10$). Thus, we demonstrated that the contents of the poison sac (primarily venom alkaloids) were not responsible for the alarm response.

Orientation Bioassay. The orientation bioassay results are shown in Figure 5. All hexane rinse samples (wire and vial, $N = 5$) from electrically stimulated ants gave positive orientation bioassay results. The assay provides a "yes" or "no" answer to orientation activity. We calculated the DE by comparing electrically stimulated ant sample (wire and vial hexane rinses) bioassay results with results from Dufour's gland extracts at known volume and DEs. Both electrically stimulated ant samples and the Dufour's gland samples were systematically diluted until orientation activity was lost. The mean last positive bioassay for the Dufour's gland samples was 0.0015 DE (SE $= \pm 0.0005$). This corresponds to ca. 0.9 pg/cm

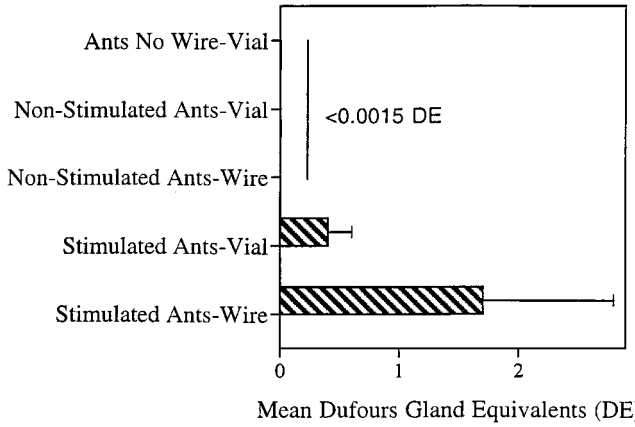


FIG. 5. Results from a recruitment orientation bioassay. Serial dilutions of treatment samples were carried out until the samples no longer gave positive results in the orientation bioassay. These results were compared with serial dilutions of Dufour's gland extracts (the source of the pheromone) containing known Dufour's gland equivalents (DE). The number of DE could then be calculated for each of the treatment samples. Only electrically stimulated workers released detectable quantities of the orientation pheromone. All other samples had to have <0.0015 DE—the lowest detectable amount. Results are shown as the mean and SE of 5 replicates.

of (*Z*,*E*)-α-farnesene, the recruitment orientation pheromone (Vander Meer et al., 1981). Knowing the starting volume of the electrically stimulated ant samples and monitoring their systematic dilution (or initial concentration) to the last positive orientation bioassay response, we related this to the results with the Dufour's gland extracts and calculated the DE of each sample (Figure 5). The initial bioassay of the controls (vial and wire rinses from samples that had ants, wire inserted, but not electrically stimulated) were negative even after fivefold concentration; therefore, they had to contain <0.0015 DE (Figure 5). Similar results were obtained with vial samples that had ants but no wire inserted (Figure 5). Like the venom alkaloids, more Dufour's gland products were deposited on the wire than on in the vial, although there was no significant difference between the two sets of samples (wire, 1.7 ± 1.1 DE vial, 0.4 ± 0.2 DE; $df = 8$, $t = 1.094$, $P = 0.359$; $N = 5$).

Olfactometer Bioassay. Each wire hexane rinse ($N = 5$) was tested in the olfactometer bioassay three times. Chi-squared analysis of the two-choice olfactometer showed that each of the wire hexane rinse replicates was attractive to worker fire ants (for each of the five replicates, $df = 1$, $\chi^2 > 3.85$, $P < 0.05$; null hypothesis of equal response to treatment and control). The mean percent of the ants responding to the treatment side of the olfactometer was 67.5 (SE = 1.5, $N = 5$). In contrast, there was not enough recruitment pheromone deposited by

electrically stimulated ants in the vial to release a response in the olfactometer bioassay ($df = 1$, $\chi^2 < 3.85$, $P > 0.05$). The mean percent of ants responding to the treatment side of the olfactometer was 56.7 (SE = 1.1, $N = 5$). The response of worker ants to the queen poison sac extract positive control was $75.5 \pm 2.6\%$ ($N = 5$, 0.33 queen equivalents/10μl hexane).

DISCUSSION

Fire ants use a wide array of semiochemicals for defense and intraspecific communication (Vander Meer and Alonso, 1998). Our results demonstrate that electrically shocked fire ants indiscriminately release exocrine gland products. Venom alkaloids from the poison sac and recruitment pheromone from the Dufour's gland were released through the sting apparatus. Alarm pheromone was released, presumably, from mandibular glands (Alonso and Vander Meer, 1997). Although not considered an exocrine gland, postpharyngeal gland contents can be regurgitated through the mouth during trophallaxis (Soroker et al., 1994). Hydrocarbons are the major class of compound found in the postpharyngeal gland, and these compounds are easily analyzed by GC (Lok et al., 1975; Vander Meer et al, 1985). Chemical analysis for hydrocarbons specific to *S. invicta* (Ross et al., 1987) from electrically stimulated fire ant workers failed to show release of postpharyngeal gland products. Release of exocrine gland products from other glandular sources, e.g., maxillary and labial glands, or the hindgut are probable, but not detectable due to our lack of knowledge regarding the behaviors and chemistry associated with these potential semiochemical sources. Interestingly, by the end of the electrical stimulation period, workers that had not been directly affected by the electrical current were inactively aggregated away from the electrified wire. This behavior may have been released by an unknown pheromone or was the result of exposure to high concentrations of known semiochemicals, e.g., venom alkaloids.

Our previous investigations into the behaviors associated with fire ant mating flights (Obin and Vander Meer, 1994; Alonso and Vander Meer, 1997) showed that the alate excitant pheromone used to initiate mating flights was produced by the mandibular glands. The products were further shown to be extremely short-lived and were released in small quantities and/or were of high volatility. Similarly, an alarm pheromone response was released from the crushed heads of workers (Wilson, 1962) and was reproducible in our laboratory (R.K.V.M., unpublished data). Our work here supports the ephemeral nature of the fire ant alarm pheromone, which, to be effective, must quickly dissipate when the threat is no longer detected (see alarm pheromone review in Vander Meer and Alonso, 1998).

Release of recruitment and alarm pheromones by an electrically stimulated worker has direct implications toward the accumulation of fire ants in electrical equipment. The estimated number of worker Dufour's gland equivalents released

by electrically stimulated fire ant workers was almost 1000 times the amount needed for significant worker attraction in the olfactometer (Vander Meer, 1986; Vander Meer et al., 1988) described here. In addition, one of the behaviors associated with alarm pheromones is attraction toward the worker releasing the alarm pheromone (Vander Meer and Alonso, 1998). Taken together, it is reasonable to assume that other fire ant workers in the vicinity of the semiochemical-releasing worker would be drawn toward the live contacts.

There are many species of ants that maintain large populations (see Hölldobler and Wilson, 1990), but none rival the high population densities of *S. invicta* in the United States. After its accidental importation into the United States in the 1930s (Lofgren, 1986), the population densities of the ant are about five times greater than found in their native South America (Porter et al., 1992), and in the United States can be as high as 100–120 mature monogyne colonies per hectare. Each colony maintains up to 250,000 workers or 30 million ants per hectare (3000 worker ants/m^2). Even if one assumes that only 5–10% of the workers are foraging, there are still 150–300 workers/m^2. Thus, it is likely that a foraging worker accidentally bridges live contacts in electrical equipment, releasing pheromones that attract nearby workers to the live contacts. These workers, in turn, release exocrine gland products, which increase pheromone concentration and the probability of attracting additional workers to the live contacts. We believe that the high population densities and the "weedy" nature (Tschinkel, 1987) of *S. invicta* is why this ant presents such a significant problem relative to electrical equipment.

Earlier work investigated the effect of electrical fields on *S. invicta* and several other ant genera (MacKay et al., 1992a,b). The authors concluded that workers from all species tested ($N = 10$) were attracted to electrical fields and that the number of ants accumulated at the electrical contacts increased with increasing voltage (directly related to electrical field strength). Our results for the fire ant present a pheromone-based alternative to their potential attraction to electric fields. All ant species are social and have evolved pheromone systems that help to maintain the health and social structure of the colony (see Vander Meer and Alonso, 1998; Hölldobler and Wilson, 1990). Therefore, there is a high probability that other ant species will also release exocrine gland products in response to electrical stimulation, which would then explain their apparent attraction to electrical fields (MacKay et al., 1992b). The differences in our conclusions may be based in semantics, since MacKay et al. (1992a) state in their discussion that while the ants accumulated at the electrically activated disks, "visual observations did not suggest the ants oriented to the powered disks." Orientation of the workers toward the powered disks would constitute "attraction," not simply that there were a number of ants at the disk after 10 min (see Vander Meer and Morel, 1988; Vander Meer and Alonso, 1998). Thus, our results support the report by Slowik et al. (1996) that electrically stimulated fire ants are not attracted to electrical fields, but may release pheromones that result in aggregations of ants at the site of electrical stimulation.

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1-OCTEN-3-OL TOGETHER WITH GEOSMIN: NEW SECRETION COMPOUNDS FROM A POLYDESMID MILLIPEDE, *Niponia nodulosa*

HISASHI ÔMURA,[1,3] YASUMASA KUWAHARA,[1,*] and TSUTOMU TANABE[2]

[1]*Laboratory of Chemical Ecology*
Division of Applied Life Sciences
Graduate School of Agriculture, Kyoto University
Sakyo-ku, Kyoto 606-8502, Japan

[2]*Tokushima Prefectural Museum*
Tokushima 770-8070, Japan

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Abstract—The small millipede *Niponia nodulosa* (Polydesmida: Cryptodesmidae) emits an earthy smell when disturbed. This smell was obtained from hexane extracts from both sexes at the sixth and seventh instars and adult stages, and was found by GC/MS analyses to be composed of two compounds, 1-octen-3-ol as the major component and geosmin as the minor component. This is the first report of these compounds in secreted substances from millipedes. As they showed little repellent activity against foraging ants in bioassays, their biological function as defensive allomones was not clearly elucidated. However, both compounds provoked negative chemotaxis of conspecific millipedes in a Y-tube olfactometer, especially 1-octen-3-ol, which was active at a dose equivalent to the content of a single millipede. Such biological activity suggests that this compound is involved in intraspecific communication in this millipede and may play a role as an alarm pheromone.

Key Words—Polydesmid millipede, *Niponia nodulosa*, secretion substances, 1-octen-3-ol, geosmin, Y-tube olfactometer, repellency, alarm pheromone.

INTRODUCTION

When disturbed, most millipedes discharge defensive secretions from body-segment glands. These secretions have been studied in a wide variety of millipedes

[3]Present address: Division of Environmental Sciences, Faculty of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739-8521, Japan.
*To whom correspondence should be addressed. E-mail: kuwa34@kais.kyoto-u.ac.jp

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and show order specificity in the distribution of major components such as hydrogen cyanide, phenols, 1,4-benzoquinones, and alkaloids (Eisner et al., 1978). For example, the order Polydesmida includes millipedes that produce hydrogen cyanide, benzaldehyde (Blum and Woodring, 1962), and mandelonitrile, regarded as the precursor of these substances (Mori et al., 1995; Noguchi et al., 1997b; Ômura et al., 2002). In addition, particular polydesmid species also emit phenol derivatives (Blum et al., 1973; Duffey et al., 1977; Mori et al., 1994; Noguchi et al., 1997a). The orders Spirobolida and Spirostreptida emit secretions containing mainly 1,4-benzoquinone derivatives (Monro et al., 1962), whereas secretions from the orders Glomerida and Polyzoniida contain characteristic alkaloids (Meinwald et al., 1966; Smolanoff et al., 1975; Wood et al., 2000). These substances are adhesive, irritating, or toxic and contribute to chemical defense against various predators (Eisner and Meinwald, 1966; Pasteels et al., 1983). However, their biological functions, except as chemical defense systems, have not been examined in detail.

Niponia nodulosa Verhoeff (Polydesmida: Cryptodesmidae, *makuragi yasude* in Japanese) is distributed in Japan (Honshu, Kyushu, and Okinawa islands) and Formosa and shows gregarious inhabitation among fallen leaves in deciduous broad-leaved forests (Shinohara and Tanabe, 1999). When disturbed, this species emits an earthy smell, which is different from that of other polydesmid secretions previously described. The present study was conducted to clarify the chemical composition of the millipede secretion and to examine the potential bioactivities of the secretion substances.

METHODS AND MATERIALS

Millipedes. N. nodulosa at nymphal (sixth and seventh instar) and adult stages were collected from litter around fallen timber of deciduous broad-leaved trees in the botanical gardens of Kyoto University of July to September 2000. Collected millipedes were reared in the laboratory for at least 10 days by feeding litter at 25°C under a 16L:8D photoperiod in plastic Petri dishes (9 cm OD $\times$ 2 cm height). Petri dishes were kept humid by spraying with water every other day.

Extraction of Millipedes. Millipedes were sexed, their body length and weight measured with minimal disturbance, and then separately soaked in hexane (150 or 200 μl for adults, and 50 μl for nymphs) for 3 min. These extracts with the characteristic earthy odor were collected by decantation and subjected to chemical analyses.

Chemical Analyses. Crude extracts were analyzed by gas chromatography (GC) and by mass spectrometry coupled with gas chromatography (GC-MS). GC analyses were carried out on a Hewlett Packard HP6850A instrument equipped with a flame ionization detector, using an HP-5 fused-silica capillary column (0.25 mm ID $\times$ 30 m, 0.25-μm film thickness). Oven temperature was programmed from 60°C to 290°C at 10°C/min with an initial 2 min hold at 60°C. Samples were

injected in splitless mode at 240°C using He as the carrier gas. Chromatograms were processed using an HP3395 integrator. GC-MS analyses were performed at 70 eV using an HP5989B mass spectrometer coupled with an HP5890 series II plus GC in splitless mode, using the same capillary column and the same analytical conditions as described above. Identification of components was based on comparison of GC retention data and mass spectra with those of authentic samples. Each component was quantified using *n*-hexadecane as the internal standard.

Ants Bioassay. To assess the effect of the millipede secretion, identified components were subjected to a repellent bioassay using ants, *Pristomyrmex pungens* Mayr (Hymenoptera: Formicidae). A block of granulated sugar (average size: 3.2 × 2.7 × 1.3 mm) was glued on a cover glass (18 × 18 mm, 0.15-mm thickness) and baited with the authentic chemical dissolved in dichloromethane. After evaporation of the solvent by air-drying, treated bait was exposed to wild foraging ants in a greenhouse at 27°C between 14:00 and 16:00 hr. Numbers of ants foraging on the treated bait and those avoiding the bait were counted for 15 min. The repellent activity at a given dose was expressed as a percentage between numbers of foraging and of avoiding ants. Tests at each dose were replicated three times using different baits. Treatment with the solvent alone had no repellent effect on ants.

Bioassay of Conspecific Millipedes. To evaluate the intraspecific biological function of the millipede secretion, chemotaxis was examined with seventh instar nymphs and adults using a transparent Y-tube olfactometer (Figure 1; a 40-mm

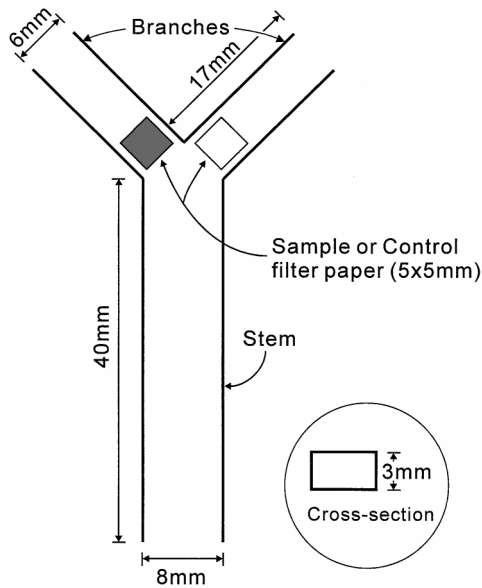


FIG. 1. Schematic drawing of the Y-tube olfactometer used for millipede bioassay.

stem 8 mm wide and 3 mm in height cross section, and two 17-mm branches 6 mm wide and 3 mm in height cross section). Bioassays were conducted in the laboratory at 25°C under illumination with an 18-W fluorescent light. Small pieces of filter paper (5 × 5 mm) were treated with either authentic chemicals dissolved in dichloromethane (sample) or with the dichloromethane alone (control). After removal of the solvent by evaporation at room temperature, one sample and one control were randomly placed into each branch of the olfactometer. To imitate the soil environment, the atmosphere in the olfactometer was kept without airflow. Millipedes were individually introduced into the olfactometer from the stem and were observed to advance. Although most millipedes advanced toward either the sample or control branch, several stopped a short distance from the junction and backed to the entrance of stem. In cases where both branches contained the control, no backward locomotion was observed. Therefore, it was reasonable to assume that the response was caused by the odor of the test compound. Each millipede was tested five times using different filter papers treated with the same dose of compound, and the frequencies of locomotion toward the sample or control or backward locomotion were recorded. The olfactometer was cleaned with ethanol and air-dried between replicates. The responsiveness of each millipede was quantified based on the two criteria: percentage of backward locomotion in five trials, and repellency index defined by the following equation,

$$\text{Repellency index} = \frac{(F_c - F_s)}{(F_c + F_s)}$$

where F_c is the frequency of locomotion toward the control, and F_s denotes the frequency of locomotion toward the sample. An index of 1 represents no choice to sample, while that of -1 represents no choice to control. Test chemicals at respective doses were examined using seven individuals of each sex at the same stage.

Test Chemicals. Authentic chemicals were purchased from commercial sources (Tokyo Chemical Industry and Wako Pure Chemical Industry). Their purities were assessed by GC and found to be above 97%.

RESULTS

Chemical Composition of Millipede Secretion. The millipede extract had two peaks (**A** and **B**); a major peak **A** at t_R 5.33 min and a minor peak **B** at t_R 12.13 min on GC analyses (Figure 2). The major component had a base ion at m/z (relative intensity, %) 57 (100) and the following diagnostic ions at m/z (%) 99 (6), 85 (11), 72 (19), and 43 (19), although no apparent M^+ ion was observed. The minor component gave an M^+ ion peak at m/z (·) (%) 182 (8), base ion at m/z (%) 112 (100), and the diagnostic ions at m/z (%) 149 (14), 125 (16), 97 (12), 83 (9),

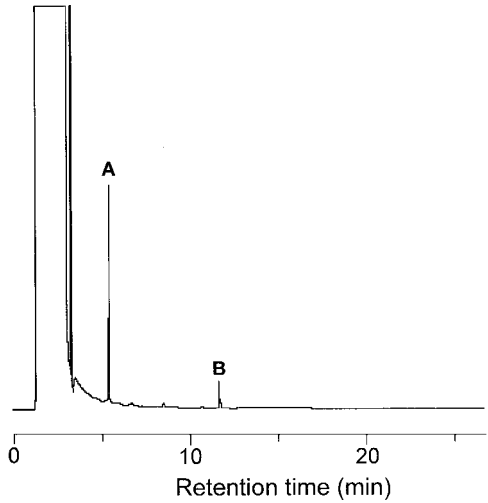


FIG. 2. A typical gas chromatogram of the hexane extract of an adult millipede, *Niponia nodulosa*. See text for the analytical conditions. Peak A, 1-octen-3-ol; peak B, geosmin. The large peak at t_R 3.2 min was noise.

67 (8), 55 (13), and 41 (9). By comparison with authentic samples, **A** and **B** were identified as 1-octen-3-ol and geosmin, respectively.

The amounts of secretory components varied with the developmental stage of millipedes (Table 1). 1-Octen-3-ol was present in approximately equal amounts in the two nymphal stages, while the average amount in adults was three fold

TABLE 1. CHEMICAL COMPOSITIONS OF HEXANE EXTRACTS FROM THREE STAGES OF *Niponia nodulosa*

Stage and sex	Body length (mm ± SD)	Body weight (mg ± SD)	Amount per millipede (ng ± SD)[a]	
			1-Octen-3-ol	Geosmin
6th instar nymph				
Male	7.8 ± 0.9	8.4 ± 2.1	127.5 ± 30.4 (1.52)[b]	7.6 ± 4.6
Female	8.4 ± 0.5	9.4 ± 0.9	120.2 ± 33.7 (1.28)	7.7 ± 4.4
7th instar nymph				
Male	11.9 ± 0.3	23.5 ± 1.6	125.7 ± 26.3 (0.53)	19.5 ± 8.6
Female	12.5 ± 0.5	26.5 ± 1.7	137.1 ± 65.1 (0.52)	16.5 ± 9.7
Adult				
Male	17.4 ± 0.8	54.4 ± 8.4	450.1 ± 179.4 (0.83)	38.7 ± 21.1
Female	17.6 ± 0.4	62.2 ± 3.3	481.0 ± 126.8 (0.77)	19.0 ± 10.4

[a] $N = 6$.
[b] Amount of 1-octen-3-ol as a percentage of body weight.

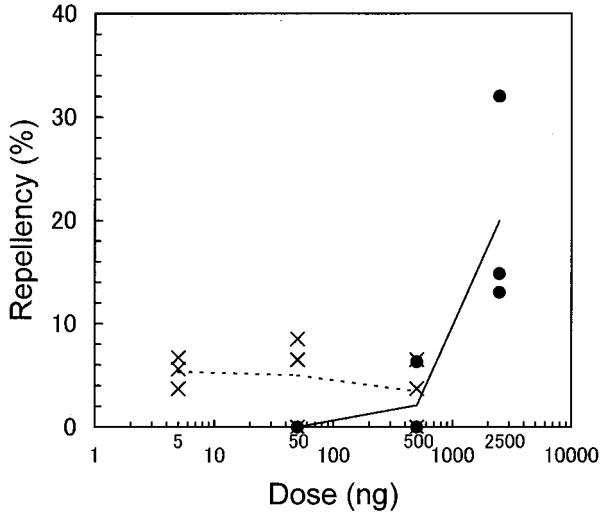


FIG. 3. The repellent effects of 1-octen-3-ol (•) and geosmin (×) on wild ants, *P. pungens*, evaluated using blocks of granulated sugar. The solid line denotes the dose–response curve for 1-octen-3-ol, while the broken line represents that for geosmin.

greater than in nymphs. This component comprised 0.5–1.5% of the body weight and reached the highest percentage by weight in sixth instar nymphs. In contrast, geosmin was detected in trace amounts and tended to increase with developmental stage. No significant differences were observed between the sexes in amount of either component at the same developmental stage (*t* test).

Repellency to Ants. Neither 1-octen-3-ol nor geosmin was repellent to ants (Figure 3). Upon exposure to the potential bait, most ants were observed to forage immediately, indicating that it had little contact repellency. In addition, remote repellent activity was vague at a short distance from the bait. 1-Octen-3-ol was slightly active (19.9%) at a dose of 2500 ng, which is approximately five fold higher than the average adult content. Geosmin was inactive within the dose range of 5–500 ng.

Repellency to Conspecific Millipedes. 1-Octen-3-ol stimulated negative chemotaxis of *N. nodulosa* at a dose equivalent to the average secretory content in millipedes (Figure 4). Although the frequency of backward locomotion was variable, this compound was slightly effective at a dose of 100 ng (14.3% on average). The average repellency index was moderate (20.2) at a dose of 100 ng and was elevated to 64.6 at a dose of 2500 ng, where this millipede preferred the odorless control to the test sample ($P < 0.05$, Wilcoxon's signed-ranks test). These results suggest that 1-octen-3-ol is repellent to consepecific millipedes. No significant differences were observed among the sexes (Mann-Whitney *U* test).

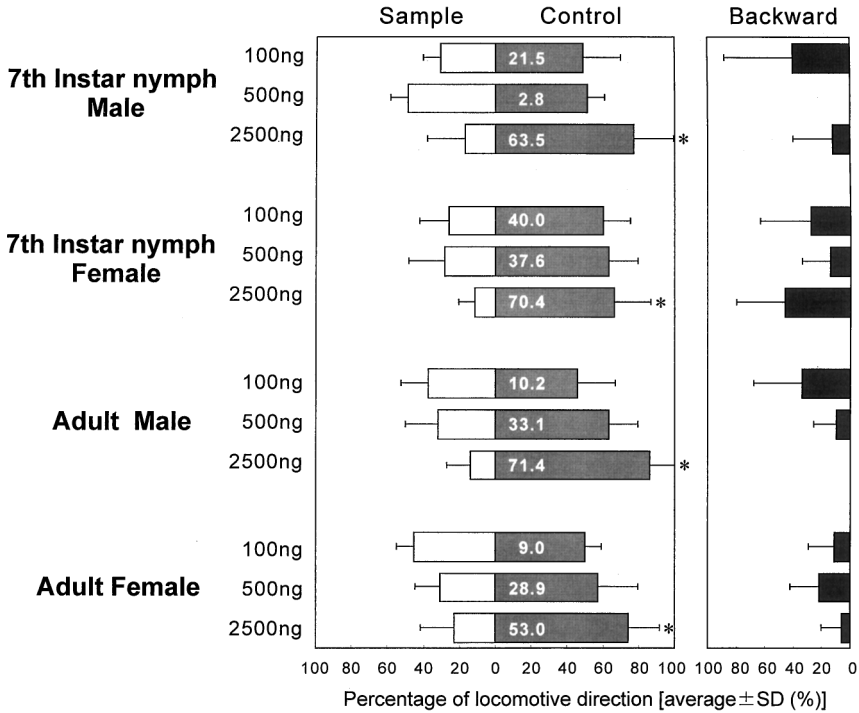


FIG. 4. Effects of 1-octen-3-ol on the chemotaxis of *N. nodulosa* in bioassays using a Y-tube olfactometer. White, gray, and black columns indicate the percentages of millipede locomotion toward sample, control, and backward locomotion, respectively. White figures on the gray columns represent repellency indexes (see text) at the respective dose of sample. Significant differences between samples and control are indicated by asterisks ($^*P < 0.05$ by Wilcoxon's signed-ranks test).

Geosmin induced backward locomotion (31.5%) and a high repellency index (84.0) at a dose of 250 ng (Figure 5: $P < 0.05$, Wilcoxon's signed-ranks test). Although the high dosage showed negative chemotaxis for *N. nodulosa*, this compound was inactive at lower doses (10 and 50 ng), equivalent to the contents of a single millipede. No significant differences were observed among the sexes (Mann-Whitney *U* test).

DISCUSSION

The secretion from *N. nodulosa* was composed of two compounds, 1-octen-3-ol as the major component and geosmin as the minor component. Neither compound has been previously reported from millipede secretions. The amount of

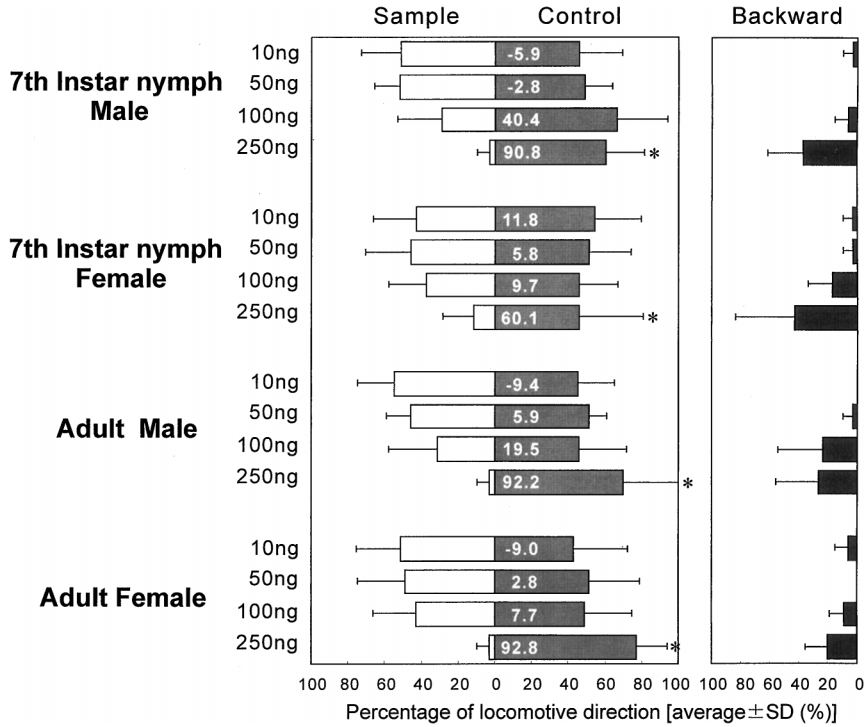


FIG. 5. Effects of geosmin on the chemotaxis of *N. nodulosa* in bioassays using a Y-tube olfactometer. White, gray, and black columns indicate the percentages of millipede locomotion toward sample, control, and backward locomotion, respectively. White figures on the gray columns represent repellency indexes (see text) at the respective dose of sample. Significant differences between samples and control are indicated by asterisks ($^*P < 0.05$ by Wilcoxon's signed-ranks test).

1-octen-3-ol was in the range of 0.5–1.5% of body weight, which is considerably greater than those of the major components (range of 0.1–0.2%) in other Japanese polydesmids (Mori et al., 1994, 1995; Noguchi et al., 1997a,b; Kuwahara et al., 2002; Ômura et al., 2002). This was deemed to be characteristic of *N. nodulosa*.

1-Octen-3-ol was first identified in the odor of Japanese matsutake mushroom (Murahashi, 1936, 1938) and has been well documented as a major volatile substance in various mushrooms (Pyysalo, 1976; Rapior et al., 1996a,b, 1997; Beltran-Garcia et al., 1997). In mushrooms, 1-octen-3-ol is biosynthesized by the enzymatic cleavage of linoleic acid (Wurzenberger and Werner, 1982; Assaf et al., 1995). Geosmin is an earthy-smelling substance and has been isolated from actinomycetes (Gerber and Lechevalier, 1965), cyanobacteria (Medsker et al., 1968), and mushrooms (Breheret et al., 1999). Bentley and Meganathan (1981) reported

that geosmin in streptomycetes is biosynthesized via the isoprenoid pathway. Both substances are regarded as microbial volatile organic compounds (MVOCs) (Jelen and Wasowicz, 1998; Fischer et al., 1999). Therefore, we speculated that these substances in *N. nodulosa* were sequestered from fungi or biosynthesized from litter in the diet. In our preliminary analyses, however, neither compound was detected from litter in the millipede habitat. To determine the origins of these substances in millipedes, further detailed investigations are required.

Most polydesmid millipedes possess chemical defense systems and secrete hydrogen cyanide and benzaldehyde as common defense substances (Towers et al., 1972; Duffey et al., 1974, 1977; Duffey and Towers, 1978). However, particular species such as *Euryurus australis* (Duffey et al., 1974) and *Eucondylodesmus elegans* (Kuwahara et al., 2002) utilize other secretory substances for chemical defense. The observation that *N. nodulosa* has different secretory components from other species indicates a diversity of defensive systems in polydesmid millipedes.

To assess the effects of defensive substances, bioassays are generally conducted using ants (e.g., Smolanoff et al., 1975; Kuwahara et al., 2002). In the present study, the secretory substances of *N. nodulosa* exerted little repellency against foraging wild ants. Although the ants tested are not a natural predator for *N. nodulosa*, it is possible that this millipede secretion is inactive toward ants. In contrast, the two secreted substances stimulated negative chemotaxis in conspecific millipedes; in particular, 1-octen-3-ol was active at a dose equivalent to the content of a single adult. On the basis of these results, we speculate on two possible roles for the millipede secretion.

The first is as a defensive allomone. Recently, 1-octen-3-ol was reported to act as a slug antifeedant (Wood et al., 2001) and as a sensory irritant for mice (Korpi et al., 1999). Moreover, MVOCs such as 1-octen-3-ol and geosmin are indicators of food spoilage (Jelen and Wasowicz, 1998; Fischer, 1999; Schnürer et al., 1999). Upon exposure to these volatile substances, predators may recognize potential health hazards and avoid the source. Therefore, it is likely that the secretory substances in *N. nodulosa* repel other potential predators (e.g., carbides, earwigs, and spiders), although little information is available regarding natural predators of this species. As Beltran-Garcia et al. (1997) reported that 1-octen-3-ol in mushroom shows antifungal activities similar to several secretory substances of polydesmid millipedes (Roncadori et al., 1985), this substance in *N. nodulosa* may serve a role in chemical defense against harmful microbes.

The other potential role is as a chemical messenger mediating intraspecific communication (pheromone). Some social or gregarious insects have evolved the capacity to use their defensive allomones as alarm pheromones (Pasteels et al., 1983). The species of millipede examined in the present study is gregarious in nature, and colonies consist of a mixture of all developmental stages in summer. We postulate one possible role of the secretory substance is as an alarm pheromone based on the following four observations: (1) the active compound originates from

the species; (2) the avoidance behavior of the millipede is reproducible upon exposure to the compound; (3) the activity is seen on exposure within the content range of a single individual; and (4) the compound is emitted in response to disturbance. However, the present repellent effect was weaker than those reported previously for alarm pheromones in other arthropods. This might have been because the conditions in our bioassay differed from those in nature, or it may suggest another potential role, e.g., as an epideictic pheromone in a gregarious population.

Millipede secretions have attracted a great deal of attention due to their interspecific roles as defensive allomones. Although intraspecific effects were suggested by the characteristics of millipedes such as soil microhabitat, gregarious population, and migration behavior known as outbreak, there have been few studies of such potential effects. The present study indicates the potential for such effects in *N. nodulosa*, and further investigation should be conducted to elucidate the ecological function of the allomones produced by this species.

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SEMIOCHEMICALS AND SOCIAL SIGNALING IN THE WILD EUROPEAN RABBIT IN AUSTRALIA: II. VARIATIONS IN CHEMICAL COMPOSITION OF CHIN GLAND SECRETION ACROSS SAMPLING SITES

R. A. HAYES,* B. J. RICHARDSON, S. C. CLAUS, and S. G. WYLLIE

Centre for Biostructural and Biomolecular Research
University of Western Sydney, Hawkesbury
Locked bag 1797, Penrith South Delivery Centre
New South Wales 1797, Australia

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Abstract—The volatile components of the chin gland secretion of the wild European rabbit, *Oryctolagus cuniculus* (L.), were investigated with the use of gas chromatography. Studies of the chemical nature of this secretion by previous workers demonstrated that it was important in the maintenance of social structure in this species. This study identified 34 different volatile components that consist primarily of aromatic and aliphatic hydrocarbons. Especially common are a series of alkyl-substituted benzene derivatives that provide most of the compound diversity in the secretion. Samples of chin gland secretion collected from animals at three different geographical locations, separated by more than 100 km, showed significant differences in composition. This work suggests that variation among populations needs to be considered when undertaking semiochemical research. Alternate nonparametric methods are also used for the analysis of chromatographic data.

Key Words—Rabbit, *Oryctolagus cuniculus*, social structure, glandular secretion, gas chromatography, mass spectrometry, behavior, chemolfactory communication multidimensional scaling, variation.

INTRODUCTION

The chemical constituents of mammal scent gland secretions have been studied in a wide variety of species. Although there are large differences among the suite of compounds occuring in various species, there is also some commonality. Many of

* To whom correspondence should be addressed. E-mail: r.hayes@uws.edu.au

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the components are aromatic, or even terpenic (Brownlee et al., 1969; Thiessen et al., 1974; Raymer et al., 1986; Novotny et al., 1990; Salamon, 1994; Perrin et al., 1996). The most common pattern is a mixture of aromatic and aliphatic compounds of varying chain length. The functional groups that appear to be most important are the oxygenated ones, especially the carboxylic acids, aldehydes, and ketones.

Almost all semiochemical studies have concentrated on samples collected from a species at only one location and at one time of the year and have interpreted the analysis of such collections as the defining semiochemical signal of the species. There are few studies of terrestrial species that consider animals from multiple sites. When such an analysis has been performed (e.g., Cantoni et al., 1996), the study has been of only one sex. However, there is no reason to assume that there are no changes in the secretions produced by animals from different places.

The primary method used to determine the chemical components of mammalian scent gland secretions is gas chromatography–mass spectrometry (GC-MS). In samples that contain a large number of compounds, it is often not merely the presence or absence of a few compounds that is important, but also the relative amounts of each component. The common method of visual comparison between chromatograms becomes impossible with this type of data set.

The majority of studies that use a statistical method for comparing chromatograms use principal components analysis (PCA) (e.g., Dudzinski, 1974; Dudzinski et al., 1975; Mykytowycz and Hesterman, 1975; Kryger, 1981; Smith et al., 1985; Belcher et al., 1986; Natynczuk and MacDonald, 1994; Salamon and Davies, 1998; Stewart et al., 1999; Lawson et al., 2000). In this type of analysis, the data array is thought of as defining the positions of samples in relation to axes that represent the full set of peaks in the chromatogram, one axis for each peak. Typically, there are many peaks, so the samples are points in a high-dimensional space. In PCA, the axes are moved to include the maximum amount of variation and variables in the minimum number of new axes. There are two major problems in the use of PCA. The first assumes that the data are normally distributed and secondly that the distances between points may become distorted when projected (Clarke and Warwick, 1994a).

Chromatographic data bear some similarity to ecological data in that both typically can be dominated by zero entries. As well, the nonzero counts show large variation and highly right-skewed distributions (Clarke and Warwick, 1994a). Informal display methods, such as clustering or multidimensional scaling (MDS) ordination, may be more valid statistical approaches, as they do not assume normal distributions. MDS works on a sample dissimilarity matrix, not on the original data array. Fewer assumptions are made about the nature and quality of the data when using MDS than with any other ordination method. Thus, MDS can provide a more realistic picture of the data. The main advantage of MDS is its greater ability to represent more complex relations accurately in low-dimensional space (Clarke and Warwick, 1994a).

To determine whether clumps of individuals are significantly different from each other, an analysis of similarity (ANOSIM) may be used. The ANOSIM tests are a range of Mantel-type permutation of randomization procedures, making no distributional assumptions. These tests depend only upon rank similarities, and thus, are, able to deal with this type of data. A SIMPER analysis can also be used to determine which peaks are most important in contributing to any differences among sites and also in determining how similar individuals are within each site. A coefficient of dispersion, calculated for these data, is a measure of dispersion based on the difference in average distance among replicate samples for a group in an MDS configuration.

The European rabbit [*Oryctolagus cuniculus* (L.)] is a species that previously has been studied semiochemically. The social structure of the rabbit is maintained in the field by the use of scent marks; including glandular secretions (Mykytowycz, 1966a–c, 1968; Hayes et al., 2002; fecal mounds (Mykytowycz and Gambale, 1969), and urine (Bell, 1981). The secretion of the submandibular cutaneous (chin) gland has been correlated especially with the maintenance of social structure. The chin glands of dominant male rabbits have a higher secretory activity than those of other individuals within the social group (Mykytowycz and Dudzinski, 1966), and these animals also show more scent-marking behavior than do other rabbits (Mykytowycz, 1968; Verberne and Blom, 1981; González-Mariscal et al., 1993). At least one of the components of the chin gland secretion is found only in the secretion from dominant males and not in that from subordinate individuals (Hayes et al., 2001). This suite of ecological and social behavior of the rabbit makes it an appropriate species to use for semiochemical analysis.

Goodrich (1983) examined the headspace volatiles of the rabbit chin secretion and found them to be mainly of an aromatic structure (including some aldehydes, ketones, and phenyl compounds), with only a few aliphatic compounds (aldehydes, ketones, and *n*-butanoic acid). Also present in the secretion are less volatile components, such as fatty acids and proteins (Goodrich and Mykytowycz, 1972).

In the present study, a comparison, using MDS and ANOSIM, was made among samples collected at three different, geographically well-separated sites to ascertain whether there was variation in the secretion throughout the range of the rabbit in Australia.

METHODS AND MATERIALS

Samples were collected from animals living at three different locations throughout east central New South Wales: Hope Farm, Cattai National Park (150°52′E, 33°33′S) located on the east bank of the Hawkesbury River in the west of Sydney (Hope Farm samples) ($N = 43$), Oaky Creek (142°22′E, 33°25′S), a NSW Agriculture study site between Bathurst and Orange in the central tablelands of New South Wales (Bathurst samples) ($N = 18$), and field enclosures of the Commonwealth

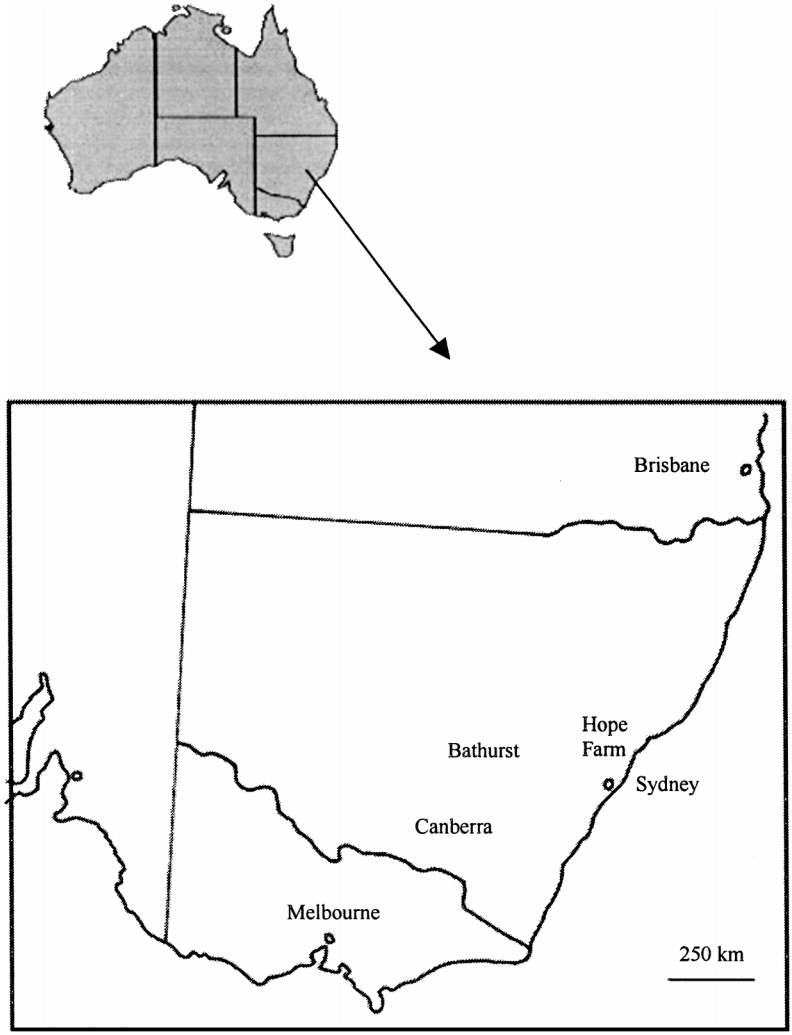


FIG. 1. A map of the southeastern portion of mainland Australia showing the location of the three sites used for sample collection of swabs of rabbit chin gland secretion.

Scientific and Industrial Research Organisation (CSIRO), Division of Wildlife and Ecology at Gungahlin (149°8′E, 35°15′S) near Canberra (Canberra samples) ($N = 22$) (Figure 1).

Wire box traps were placed near entrances to warrens that were in active use. Chin gland secretions were collected from the animals live-trapped in the field. To

collect the secretion, an area of approximately 2 cm^2 of fur was clipped from the chin area of the rabbit with scissors. A cotton bud, previously baked at 140°C for at least 4 hr to remove volatile contaminants, was swabbed over the clipped region 20 times. The swab was placed into a headspace vial (10 ml), and the vial was closed with an airtight Teflon-faced rubber seal for analysis by dynamic headspace gas chromatography. All samples were stored in the dark in a freezer at −20°C until analyzed. Duplicate swabs were taken.

The vial was heated to 30°C in a heating block. Laboratory air (3.2 liter) was pumped through a charcoal trap, then over the sample at a flow rate of 13 ml/min. After passing over the sample, the air flowed through gas chromatography injection port liners packed with the adsorbent agent Tenax TA (35/60 mesh; (90 ± 2 μg). At the end of the collection time (246 min), the liner was placed in the injector port of a GC (HP5890 Series II). The Tenax was thermally desorbed with a programmable temperature vaporizer (PTV) (Activon).

Analysis was conducted with a Hewlett Packard 5890 Series II gas chromatograph (GC) equipped with a flame ionization detector and fitted with a silica capillary column (SGE, model BP-1, 25 m long, 0.22 mm ID and 0.1-μm film thickness). Data were acquired under the following GC conditions—inlet temperature: 200°C, carrier gas: nitrogen at 23 cm/sec, split ratio: 1:20, detector temperature: 260°C, temperature program: initial temperature 45°C, initial time 5 min, rate 8°C/min, final temperature 250°C, final time 5 min. The temperature program for the PTV was—initial temperature: 40°C, ramp rate: 16°C/sec, final temperature: 200°C.

The identities of peaks in the chromatogram were determined by use of another HP5890 GC coupled to a mass spectrometer (HP 5971A, Hewlett Packard) (GC-MS) with an attached PTV. Data were acquired under the following GC conditions—inlet temperature: 260°C, carrier gas: helium at 1.1 ml/min, split ratio 1:2, detector temperature 260°C, temperature program: initial temperature 40°C, initial time 5 min, rate 8°C/min, final temperature 250°C. The MS was held at 190°C in the ion source using an ionization energy of 70 eV and a scan rate of 0.9 scans/sec. The PTV program was—initial temperature: 35°C, ramp rate: 12°C/sec, final temperature 220°C. Peak identities were assigned by comparison with the Wiley mass spectral library. Confirmation of the identity of several compounds was obtained with authentic samples (Sigma) using the same sampling method and comparison of retention times (see Table 1 below).

An external standard hydrocarbon mixture (C_8–C_{18}) was injected into the GC and analyzed under the same conditions to enable calculation of Kovats indices.

Sample blanks were run twice every week to ensure that volatiles recorded were from the secretion and not due to the sampling procedure. The blanks consisted of an unused cotton bud analyzed by the same procedure as for the samples. The number of peaks in blank and sample swabs was compared by analysis of variance (GMAV5, 1997).

Each integrated peak in the chromatogram was assigned an arbitrary number. The numbering system was kept consistent between sample runs, so that a peak of a given number in a chromatogram corresponded to the same peak in other chromatograms. The Wiley spectral library and a Kovats analysis were used to assign identities to the numbered peaks.

The presence of peaks in the chromatogram and their relative areas were examined statistically by a nonparametric method [multidimensional scaling (MDS)] to ascertain if any differences could be detected among animals from different locations. MDS uses a similarity matrix of the data and produces a graphical representation. Each point in the MDS represents an individual rabbit, and points that are close together (clumped) correspond to individuals with similar peak composition (presence and abundance) (Clarke and Warwick, 1994b).

To determine whether clusters of individual rabbits were significantly different from each other, an analysis of similarity (ANOSIM) was used. A SIMPER analysis was used to determine which peaks were most important in contributing to any differences among sites and also how similar individuals within each site were. A coefficient of dispersion was also calculated for these data. Software used for the multivariate analysis was Primer 5 for Windows (V 5.2.4, 2001).

RESULTS

There were significantly fewer peaks detected in the chromatograms from blank swabs (1.09 ± 0.25) than in swabs from rabbits (17.4 ± 1.59) (ANOVA, $F = 206.46$, $P < 0.001$).

Compounds detected in the swabs of the chin region of the European rabbit are listed (Table 1). Ethylbenzene, phenol, benzaldehyde, acetophenone, and 4-methylphenol (*p*-cresol) have been previously identified in rabbit chin gland secretion (Goodrich, 1983). The proportion of samples containing peaks from the three sites and the average relative percentage area of the peak are also given.

The MDS analysis shows a grouping of individual samples from each site that creates three different groups (ANOSIM: Global $R = 0.724$, $P = 0.001$). The Canberra population shows a tight clump with a low dispersion coefficient (0.345) and an average similarity within the group of 80% (SIMPER). This location has a signal that is different from the other locations (ANOSIM: $R = 0.684$ and $R = 0.975$ for Bathurst and Hope Farm, respectively, $P = 0.001$).

Both Bathurst and Hope Farm have similar levels of dispersion that are greater than that for Canberra (1.301 and 1.17, respectively), with an average similarity within the group of 47 and 52%, respectively (SIMPER). Even with this greater dispersion and some overlapping of individuals, these locations are still different from each other (ANOSIM: $R = 0.491$, $P = 0.001$) (Figure 2). The Bathurst samples sorted into two groups, but this separation is not due to either age or sex.

TABLE 1. LIST OF COMPOUNDS FROM RABBIT CHIN GLAND SECRETION[a]

Peak	Kovats index	Identification	I.D. method	% of animals with peak				Average relative % area of peak		
				Bathurst	Canberra	Hope Farm	Total	Bathurst	Canberra	Hope Farm
3		unidentified[b]		50	0	95	60	13.11 ± 4.26	0.00	34.74 ± 3.33
5	685	unidentified		50	95	30	52	1.46 ± 0.36	3.63 ± 0.37	0.16 ± 0.05
6	746	unidentified		100	100	60	80	1.61 ± 0.41	0.70 ± 0.08	0.90 ± 0.22
7	766	unidentified		44	50	12	29	0.29 ± 0.14	0.13 ± 0.05	0.15 ± 0.07
10	801	unidentified		44	23	21	27	0.18 ± 0.05	0.03 ± 0.01	0.19 ± 0.07
12	837	dimethylbenzene (xylene)[b,c]	RT/MS	100	100	100	100	20.14 ± 4.60	5.83 ± 2.32	12.53 ± 1.46
13	846	ethylbenzene[b,c]	RT/MS	100	100	100	100	11.36 ± 2.12	2.71 ± 0.69	9.88 ± 1.03
14	864	1,3-dimethylbenzene (*m*-xylene)	RT/MS	56	68	16	39	0.51 ± 0.20	0.18 ± 0.03	0.28 ± 0.12
15	868	dimethylbenzene (xylene)[c]	RT/MS	100	100	100	100	1.79 ± 0.15	0.75 ± 0.17	1.90 ± 0.16
38	928	? α-pinene[c]	RT/MS	61	45	28	40	0.13 ± 0.04	0.31 ± 0.09	0.19 ± 0.07
18	955	2,4,6-trimethyloctane	MS	44	95	14	42	0.59 ± 0.18	3.34 ± 0.19	0.10 ± 0.05
19	961	phenol[c]	RT/MS	44	95	63	67	0.42 ± 0.13	3.97 ± 0.20	1.09 ± 0.31
20	964	benzaldehyde[c]	RT/MS	78	95	28	57	0.82 ± 0.14	2.40 ± 0.13	0.34 ± 0.11
40	979	unidentified		50	95	0	36	0.22 ± 0.10	0.37 ± 0.02	0.00
22	984	1,2,4-trimethylbenzene[b,c]	RT/MS	44	95	0	35	0.63 ± 0.20	6.27 ± 0.35	0.00
23	992	unidentified		39	9	0	11	0.14 ± 0.05	0.02 ± 0.01	0.00
24	995	unidentified		39	95	26	47	0.18 ± 0.06	0.97 ± 0.08	0.17 ± 0.06
25	998	? 2-ethylhexanol	MS	56	100	0	39	0.82 ± 0.20	2.43 ± 0.06	0.00
26	1004	unidentified		67	95	0	40	0.33 ± 0.11	0.39 ± 0.02	0.00
28	1017	1-methyl-4-isopropylbenzene[b]	MS	56	100	81	81	1.71 ± 0.46	17.82 ± 1.15	2.28 ± 0.30
29	1035	hydrocarbon		44	95	23	47	0.44 ± 0.15	2.17 ± 0.13	0.26 ± 0.09
30	1042	? γ-terpinene	MS	39	95	30	49	0.16 ± 0.05	0.54 ± 0.03	0.37 ± 0.15
31	1066	acetophenone[c]	RT/MS	94	95	86	90	1.43 ± 0.32	0.95 ± 0.17	2.71 ± 0.83
45	1092	2-phenoxyethanol[b]	RT/MS	50	73	19	40	6.49 ± 2.07	0.22 ± 0.06	1.94 ± 0.86
54	1150	hydrocarbon	MS	0	9	0	2	0.00	0.24 ± 0.22	0.00
46	1158	hydrocarbon	MS	0	0	2	1	0.00	0.00	0.02 ± 0.02
33	1164	hydrocarbon	MS	50	91	68	84	2.40 ± 0.96	0.61 ± 0.08	3.99 ± 0.65

TABLE 1. CONTINUED

Peak	Kovats index	Identification	I.D. method	% of animals with peak				Average relative % area of peak		
				Bathurst	Canberra	Hope Farm	Total	Bathurst	Canberra	Hope Farm
52	1183	decanal	MS	50	91	2	36	0.75 ± 0.47	0.72 ± 0.06	0.04 ± 0.04
34	1197	unidentified		17	0	19	13	0.05 ± 0.03	0.00	0.26 ± 0.12
53	1222	4-methylphenol (*p*-cresol)	RT/MS	44	77	2	31	0.22 ± 0.09	0.17 ± 0.02	0.01 ± 0.01
59	1282	? 2-methyl-5-isopropylphenol	MS	0	100	0	27	0.00	3.22 ± 0.24	0.00
58	1362	butylbenzoate	MS	33	0	70	43	0.25 ± 0.10	0.00	0.53 ± 0.08
36	1477	unidentified		94	5	77	61	1.86 ± 0.43	0.01 ± 0.01	4.38 ± 0.98
44		unidentified[b]		0	0	72	37	0.00	0.00	5.70 ± 1.50

[a] The experimentally derived values of the Kovats Index are given, as is the identification method (mass spectrum, retention time or both). The percentage of rabbits from each site [Bathurst ($N = 18$), Canberra ($N = 22$) and Hope farm ($N = 43$)] in which the peak occurs and the average relative percentage area of the peak in each sample is also shown. ? = tentative identification of the compound.

[b] Most important compounds in distinguishing between samples from each of the sites (SIMPER).

[c] Confirmed by authentic samples.

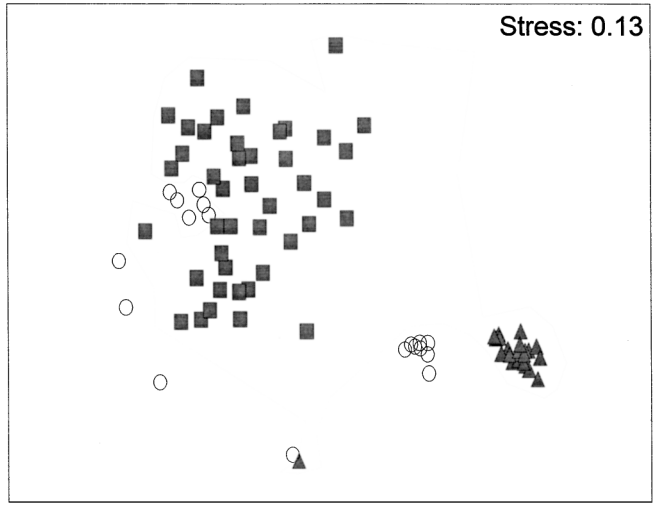


FIG. 2. Multidimensional scaling (MDS) ordination based on a Bray-Curtis similarity matrix of the three sites Bathurst (○), Canberra (▲), and Hope Farm (■) rabbits.

The majority of the differences among groups were in the relative intensities of the peaks. However, there were two incidences of an exclusive compound. Peak 59 (tentatively identified as 2-methylisopropylphenol) was found only in the swabs of rabbits from the Canberra site and was present in all animals. However, it is not important in the separation of Canberra animals from samples at the other sites. Peak 44 (unidentified) was found in 72% of individulas at Hope Farm and not in those at the other two sites. It distinguished the Bathurst and Hope Farm sites (Table 1). There are two other peaks that are specific to only one site; however, both of these were found in only one animal and are unlikely to be biologically relevant.

DISCUSSION

MDS analysis indicated that animals may be grouped according to site on the basis of compounds present in the headspace above their chin gland secretions. These differences are predominantly due to changes in the relative proportion of the peaks rather than to changes in the compounds present.

Some of the identified compounds were detected in the swabs from virtually every rabbit examined (e.g., ethylbenzene). Others were detected only in swabs from one individual of the 83 examined (e.g., peaks 54 and 49). Those compounds that were detected in the secretion of only one or two individuals are less

likely to be an important part of the semiochemical message than those that occur regularly.

There were four compounds found only in samples at one site, and one of these (peak 44) was important in distinguishing among sites. It is not clear, however, what significance they may have to the rabbits.

The between-suite variation is also within the compounds that are most commonly found in chin gland secretions, such as ethylbenzene and xylene, both of which occure in all animals swabbed, rather than in the more rare components. These differences are not due to secondary plant compounds, and, thus, do not seem to be related to diet differences. They are rather likely due to some more fundamental difference among rabbits from different places.

While the Bathurst and Canberra populations derive from the same release of rabbits 100 generations ago, the Hope Farm rabbits are at least partly derived from a different genetic stock from Europe (Stodart and Parer, 1988; Phillips et al., 2002). The differences observed among secretions found at different sites may by due to genetic differences among populations. Genetic studies using allozymes, mitochondrial DNA, and microsatellites show that there can be large differences in allele frequencies among locations separated by only a few kilometers (Richardson, 1981; Zenger, 1996; Vachot, unpublished results). Parallel changes in the genes underlying the metabolic pathways leading to the compounds secreted could form the basis of the observed changes in concentrations. The production of distinctive compounds at Canberra and Hope Farm is worthy of further study. The animals from Canberra, although kept in outdoor enclosures, have a limited ability to interbreed. The low variation in these samples may be due to a loss of heterozygosity due to higher levels of inbreeding.

An MDS analysis may allow a more thorough examination of chromatographic data than does the more commonly used PCA. PCA has little flexibility in defining dissimilarity. Ordination is essentially a technique for converting dissimilarities of chemical composition among samples into Euclidean distances between these samples in a 2- or higher dimensional plot. PCA defines dissimilarity as the Euclidean distance between two samples in the full *n*-dimensional space; however, this is a poor way to define sample dissimilarity. Having defined dissimilarity as distance in the *n*-dimensional space, PCA converts these distances by projection onto the 2- or 3-dimensional-ordination plane. This may distort some distances. Two samples that are relatively far apart on the axis representing the third principal component can end up being coincident when projected (perhaps from "opposite sides") onto the plane formed from the first two principal components (Clarke and Warwick, 1994a).

As well as obtaining a more accurate knowledge of the entire suite of compounds associated with a particular species, by using several different study sites a researcher is able to get some idea of what compounds are important. Those compounds that are conserved across different locations are more likely to from part

of a species-wide message than are those that are specific to one locality. Isolation of individuals by distance can be used to ascertain the important components of a semiochemical message.

Many of the previous semiochemical studies on mammals have been conducted on small sample sizes of organisms (<20) that have been collected from only one location (Müller-Schwarze, 1972; Black-Cleworth and Verberne, 1975; Yarger et al., 1977; Raymer et al., 1986; Apps et al., 1988; Schultze et al., 1988; Jemiolo et al., 1991; Richardson, 1991; Price and Feistner, 1994; Brown et al., 1996; Eggert et al., 1996; Rasmussen et al., 1996; Salamon and Davies, 1988). This study identified differences in the semiochemical secretion of the chin gland among rabbits from different sampling sites. Variation among animals occurs, and in order to define the semiochemicals, produced by a species it is important to examine a large number of individuals at more than one time of year and from more than one place.

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CHEMICAL POLYMORPHISM OF THE CUTICULAR LIPIDS OF THE CABBAGE WHITE *Pieris rapae*

CRISTIAN ARSENE,[1] STEFAN SCHULZ[1,*] and JOOP J. A. VAN LOON[2]

[1]*Institut für Organische Chemie, Technische Universität Braunschweig, Hagenring 30. D-38106, Braunschweig, Germany*

[2]*Laboratory of Entomology, Wageningen University, P.O. Box 8031, 6700 EH Wageningen The Netherlands*

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Abstract—The epicuticular composition of different body parts of the Cabbage White, *Pieris rapae* L., was investigated using GC and GC/MS. The major group of components, hydrocarbons, occurs in two distinct classes, which show different distributions on the cuticle of the insects. Unbranched shorter chain compounds (C_{21} to C_{31}, linear group) dominate on body, head and wings, while longer chain, polymethyl-branched compounds (C_{35} to C_{39}, branched group) are predominantly found on the antennae. Several other components like 1,3-pentacosadiene and oxygenated aliphatic compounds occur in minor amounts on the cuticle. The reason for this polymorphism is discussed.

Key Words—*Pieris rapae*, alkanes, alkenes, alkadienes, alcohols, alkanediols, cuticle, cuticular lipids, tetrahydrofurans.

INTRODUCTION

The epicuticular lipids of insects have been extensively studied by modern gas chromatographic and mass spectrometric methods, the predominant class of compounds being alkanes. Numerous reviews have been published dealing with the composition of the insect cuticle and its function as a barrier between the body and the environment or as a carrier of information (e.g., Nelson and Blomquist, 1995). Nevertheless, comparative analyses among different body parts have rarely been performed (see references cited in Howard, 1993; Buckner et al., 1994; Young et al., 2000), and antennal lipids have been neglected. During our studies on the mating system of Pierid butterflies, we found striking differences in the composition of the surface lipids of the Cabbage White, *Pieris rapae*, among different body regions.

* To whom correspondence should be addressed. E-mail: stefan.schulz@tu-bs.de

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METHODS AND MATERIALS

About 20 freshly eclosed butterflies of *P. rapae* L. (Lepidoptera: Pieridae) were killed by freezing, sexed, and dissected into wings, body (thorax, abdomen, and legs), head, and antennae. Extracts of the different body parts were obtained by a short rinse with pentane (Merck, Suprasolv). Care was taken to avoid contamination of the sample with hemolymph by not allowing contact of solvent and cut surfaces. The different extracts were subjected to GC and GC-MS analyses as previously described (Shulz, 2001). SPME analyses were performed by rubbing a 100 μm PDMS fiber, conditioned at 250°C, on all the respective body parts. This procedure was repeated twenty times to obtain enough material for analyses. All individual analyses were repeated three to five times. Derivatization with dimethyldisulfide (DMDS) was performed according to Francis and Veland (1981).

RESULTS AND DISCUSSION

The analyses revealed that hydrocarbons are the major class of lipids present on the epicuticle in all samples. They were characterized by well established methods based on the interpretation of mass spectra and gas chromatographic retention indices (Carlson et al., 1998; Schulz, 2001). Two different alkane groups could be distinguished: Straight chain alkanes ranging in chain length from C_{21} to C_{31} (linear group) and di-, tri-, and tetramethyl branched alkanes with longer chain length (C_{35} to C_{39}, branched group). In the first group, small amounts of methyl branched alkanes and also straight chain 1-alkenes co-occurred. The double bond of the alkenes is predominately located at C-1, which was proven by DMDS derivatization {e.g., characteristic ions for 1-heneicosene $m/z = 327$ (100, M-61) and 61 (21)}. This position is rarely found in insect lipids (Howard, 1993; Nelson and Blomquist, 1995). The two alkane groups showed clear differences in their abundance among different body parts. The linear group predominates on the body and the head, while it is markedly reduced on the antennae where the branched group predominates. This latter group is found in smaller amounts on the other body parts as well. On the wings, a modified linear group predominates, which is reduced in the amount of the shorter chain members (Figure 1). The two sexes did not differ in the distribution of hydrocarbons over the respective body parts, as proved by separate analyses. SPME analyses revealed that the identified compounds are present on the surface of the outer cuticle.

Unique compounds are (*E*)- and (*Z*)-1,3-pentacosadiene, not previously reported from insects, which are most abundant on the head and not accompanied by any homologs, as found with the other hydrocarbons. Several long chain oxygenated compounds like C_{29} 2,5-dialkyltetrahydrofurans (present only on the

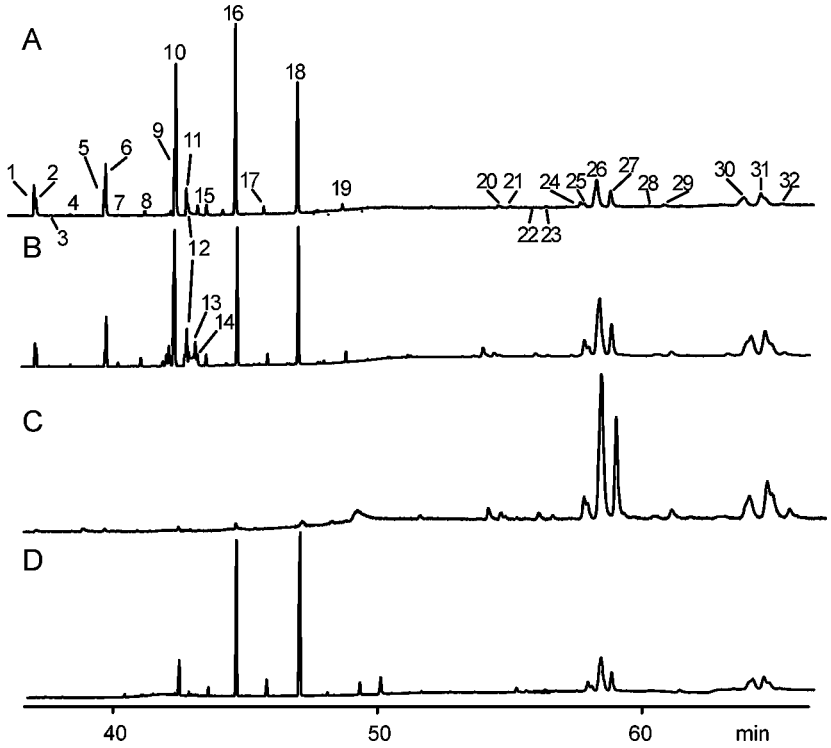


FIG. 1. Gas chromatograms of solvent extracts of cuticular lipids of *P. rapae*. A: body; B: head; C: antennae; D: wing. Numbers refer to Table 1.

antennae), alkyl methyl ethers, secondary alcohols, and corresponding 1,2-, 1,3-, and 1,4-diols, like 6,9-nonacosanediol, were also identified. They often occur in enhanced proportions on the antennae or the head.

While qualitative differences in the composition of different body parts have been observed occasionally in insects, no such large differences as found in the present study have been reported. There might be several reasons for the observed chemical polymorphism. The water permeability of the epicuticle of insects seems to depend on the melting point of the lipids (Gibbs, 2002). Because methyl branchings have a much stronger influence on the melting temperature of hydrocarbons than chain length (Gibbs, 2002)., it can be safely assumed that the branched group of hydrocarbons has a lower melting point than the linear group. This might result in higher water loss, higher fluidity, and, most importantly, also higher diffusion rates of dissolved molecules. The first event in the olfactory process is adsorption or solution of a signal molecule on the antennal cuticle. The molecule then has to travel by diffusion to pores in the sensillum surface, where it is caught by a

TABLE 1. HYDROCARBONS IDENTIFIED IN THE EPICUTICLE OF *Pieris rapaea*[a]

No.	Compound	an	he	bo	wi
1	1-henicosene	—	1.3	0.5	—
2	henicosane	0.03	1.0	0.4	0.5
3	9- and 11-methylhenicosane	—	0.1	0.02	—
4	docosane	0.01	0.1	0.03	0.1
5	1-tricosene	0.04	2.7	0.4	0.2
6	tricosane	0.2	3.7	1.3	0.9
7	9- and 11-methyltricosane	—	0.3	0.1	—
8	tetracosane	0.03	0.6	0.3	0.2
9	1-pentacosene	0.2	4.4	5.1	1.0
10	pentacosane	0.8	16.0	15.3	3.4
11	9-, 11- and 13-methylpentacosane	0.06	0.7	0.2	0.02
12	*E*- and (*Z*)-1,3-pentacosadiene	0.17	5.3	1.8	0.9
13	7,11-dimethylpentacosane	0.04	2.0	0.5	0.3
14	3-methylpentacosane	0.02	0.9	0.3	0.04
15	hexacosane	0.2	0.7	0.6	0.8
16	heptacosane	0.9	8.8	21.2	36.4
17	octacosane	0.1	0.6	0.8	1.2
18	nonacosane	0.8	8.5	16.0	21.3
19	hentriacontane	—	0.7	1.6	1.1
	sum linear group	**3.6**	**58.4**	**66.4**	**68.4**
20	11,15,19- and 13,17,21-trimethylpentatriacontane	1.7	1.3	0.7	0.9
21	11,15,19,23-tetramethylpentatriacontane	1.2	0.6	0.3	0.4
22	12,16,20-, 13,17,21- and 14,18,22-trimethylhexatriacontane	1.0	0.5	0.4	0.5
23	11,15,19,23-tetramethylhexatriacontane	0.5	0.1	0.2	0.2
24	13,17-, 15,19- and 17,21-dimethylheptatriacontane	4.4	2.4	1.3	1.8
25	11,15-dimethylheptatriacontane	2.5	1.3	1.2	1.8
26	11,15,19-, 13,17,21- and 15,19,23-trimethylheptatriacontane	34.2	11.4	9.7	9.5
27	11,15,19,23-tetramethylheptatriacontane	20.4	4.3	3.6	3.7
28	12,16-, 13,17-, 14,18-, 15,19- and 17,21-dimethyloctatriacontane	1.1	0.3	0.6	0.6
29	12,16,20- and 14,18,22-trimethyloctatriacontane	1.7	0.9	0.9	0.9
30	11,15-, 13,17-, 15,19- and 17,21-dimethylnonatriacontane	8.5	6.9	5.0	3.8
31	11,15,19-, 13,17,21- and 15,19,23-trimethylnonatriacontane	10.3	7.0	7.2	4.8
32	11,15,19,23-tetramethylnonatriacontane	4.4	1.0	0.8	0.2
	sum branched group	**91.9**	**38.0**	**31.9**	**29.1**

[a] he: head; an: antennae; bo: body; wi: wing. Numbers (%) are relative proportions within the sample. Where percentages do not add up to 100%, the remaining percentage is comprised of oxygen containing compounds.

binding protein (Kanaujia and Kaissling, 1985; Kaissling, 2001). This diffusion is certainly faster in materials with higher fluidity, a probable reason for the enhanced proportion of multi-branched hydrocarbons on the antennae. A fast transport of signal molecules on the antennal surface has been shown by Kanaujia and Kaissling (1985). Furthermore, the different patterns and singular components of the cuticular lipids may be used in chemical communication (Nelson and Blomquist, 1995), especially at close range and in the contact phase of courtship and mating behavior. This might be one reason for the presence of unique compounds on certain body surface areas. Most likely a distribution of lipids as in *P. rapae* may also be found in many other insects.

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Book Review

Insect Physiology and Biochemistry. By J. L. Nation. CRC Press, Boca Raton, Florida. $109.95. 485 pp. ISBN 0-8493-1181-0

Dramatic discoveries in molecular biology, recombinant genetic manipulation of plants and animals, and genomics, all promising remarkable advances in crop sciences, public health, and medicine, fill the daily news. Few among the public are aware of or credit many of these advances to knowledge derived from fundamental studies of insect physiology and biochemistry and especially of the genome of the fruit fly *Drosophila melanogaster*.

Studies of the form and function of insects have enjoyed a long history, yet only in the last century have investigations of the biology of insects so vastly enriched science and, by association, human welfare. The short lives of insects and their high reproductive potential permit rapid life cycle investigations that are difficult with "higher" longer lived animals. Since all eucaryotic organisms share common developmental and biochemical pathways regulated by genes, biologists, especially physiologists, evolutionary biologists, geneticists, biochemists, and molecular biologists, concentrate their investigations on insect models. Early physiology textbooks related form and function, with scant inclusion of insect biochemistry beyond obvious elements that could be borrowed from studies of human biochemistry. With time, increasing biochemical investigation targeted directly to insects, along with exploration of the genome of the fruit fly, birthed the modern disciplines of genetics and molecular biology. Equally, the information from insect science and its impact on plant and animal protection from insect pests and disease vectors, as well as the toxicological concerns emerging from worldwide pesticide usage, has sparked increased demand for appropriate courses throughout academia.

Basic formal coursework in insect physiology, once relegated nearly exclusively to students in academic departments of entomology, parasitology, vector biology, and toxicology, now command an important presence in the curriculum of nearly all-aspiring biological scientists irrespective of their interest in the fundamental or applied sciences. Advances in plant pest and disease vector control taken together with contributions of insect science to studies of biochemistry, chemical ecology, evolution, genetics, and molecular biology are commanding reasons for

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instruction focussed on the physiology and biochemistry of the Class Insecta that composes the principal animal biota of earthlife.

Textbooks serve principally to introduce students to the basics of a discipline and awareness of the expanding literature of the subject. The recently published textbook *Insect Physiology and Biochemistry* by Professor James L. Nation of the University of Florida accommodates very well the need for a contemporary introduction to the subject. Composed of fifteen chapters, the text is arranged conventionally, beginning with Embryogenesis and ending with Reproduction and an appendix that positions the Class Insecta among related taxa to familiarize the invertebrate challenged student with the status of these dominant planetary lifeforms. All chapters combine contemporary research data with excellent illustrations and recent references. Early insect physiology texts often rigorously referenced the works of pioneering contributors; some with credits even to Aristotle. Fortunately, this current work disdains such practice, favoring for the most part recent studies and interpretations. Nation has clearly set the goal of demonstrating the basic aspects of insect physiology and biochemistry to undergraduate students largely free of the complexities required by more advanced students. This is achieved in a remarkably readable and well-illustrated volume.

The first chapter Embryogenesis relates the development of primordial cells and their differentiation into tissues, then segmentation and organogenesis and their regulation by known gene groups. Elements of biochemistry enter the text with the chapters on Digestion and Nutrition and continue throughout most of the following chapters with expansion in the chapter on Intermediary Metabolism. It is noteworthy that the chemistry is introduced in close support of physiological functions. Thus, elements of chemistry highlight the function rather than dramatize the author's personal store of detailed biochemical minutiae. Would that this practice was more generally adhered to in introductory texts. The following chapters i.e., Neuroanatomy, Neurophysiology, Muscles etc. focus on the specific form and function of each organ system and are clearly presented and illustrated.

Given my personal research interests I turned directly to the chapter on Hormones and Development to obtain some flavor of the organization, clarity, and content of a chapter I felt most competent to criticize. This chapter is organized around the anatomy of the endocrine organs and details their regulation of metamorphosis and reproduction. It provides a broad overview of insect endocrines, eschewing extraneous detail and resisting review of the comparative aspects of invertebrate/vertebrate endocrinology.

The penultimate chapter on Pheromones precedes the final chapter on Reproduction and is usefully positioned to highlight the participation of chemical communication among insects for co-location of the sexes for reproduction (i.e., sex pheromones). This fascinating chapter (my favorite) clearly sets forth the various modes of chemical communication among insects, and classifies them according to the behaviors elicited (i.e., which benefits). The treatment of receptors, binding

proteins, signal transduction, biosynthesis, and pheromone blends, however brief, is quite comprehensive for an introductory treatment. Some investigational methods including electrophysiological tools and plume theory are also described. Certainly the detailed chemistry of hundreds of pheromones is known, but Nation wisely imparts details of but a few model structures. The biological relationships chosen to illustrate the elements of insect chemical communication are broad, topical, comprehensive, and composed to introduce students to this dynamic research area. I was astonished at the author's restraint being aware of his own studies and enthusiasm for the subject. Although he mentions the use of pheromones in plant and public health protection, I would have liked to see this expanded a bit for the students anticipating applied careers.

Nation has a splendid feel for his subject and his direct and lucid presentations clearly derive from his classroom lecturing experience. He concisely essays the quanta of knowledge to address the introductory needs of students, yet provides the references appropriate to lead the serious student well beyond. This is particularly true in the chapter on Nutrition, which seemed especially comprehensive. Reviewers are supposed to find and detail things they do not like about the books they judge, but I am unable to find areas of neglect or errors of fact. On the first read I would have expected an expansion on the pheromone chapter to include the author's special insights on *Tephridid* flies or a broader treatment of the burgeoning literature on the pheromones of social insects. Yet, if I stand in the author's shoes for a moment I can see that expansions on such large specialties easily become a black hole in an introductory text. The most critical comment I can make is to wish for some mention of how the study of the insect physiology and biochemistry has benefited humanity i.e., development of environmentally safe methods of plant and public health protection and advances in genetics and medicine.

Insect Physiology and Biochemistry provides an excellent introduction of the subjects to the undergraduate for whom it was written and will serve as a primer for the non-specialist eager to employ insects as their research models. The text is clear, well arranged and a pleasure to read. It presents the broad concepts and focuses on contemporary references rather than exhaustive inclusion of the contributions of the pioneering investigators of insect sciences so well documented in previous texts and monographs. In all, this textbook is an impressive work, emblematic of Nation's ability to present clearly the salient highlights of insect physiology and biochemistry. Its wide adoption as a teaching text is assured.

William S. Bowers
Department of Entomology
Laboratory of Chemical Ecology
The University of Arizona
Tucson, Arizona 85721

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ANNOUNCEMENT

FONDATION JEAN-MARIE DELWART AWARD 2003 IN ANIMAL AND HUMAN ETHOLOGY CHEMICAL COMMUNICATION

The Jean-Marie Delwart Foundation will award in 2003 a Prize for an original work or series of works, individual or collective in the field of *Chemical Communication*, dealing with the specific action of certain substances on the organisms (Invertebrates, Vertebrates including Man, Plant/Animal relations, etc.) in relation with the corresponding behavioral patterns.

The Prize, in the amount of $10.000, will be attributed to works written or translated in French or in English, which should be sent by April 15th 2003 to the following address:

Fondation Jean-Marie Delwart
Attn: Raphaëlle Holender
U.C.L. Bâtiment Pythagore
4, Place des Sciences (Bte 4)
B-1348 Louvain-la-Neuve
Belgique
E-mail: fondation.j.m.delwart@skynet.be

The applications can be submitted either by the candidates themselves or by recognized specialists in the field considered or again by academic institutions (Academies, Universities, Faculties, Departments, Centres of Research, etc.). In all cases, the applications will be accompanied by a letter of presentation, a detailed curriculum vitae and a complete list of publications (in quadruplicate). The candidates are requested to send copies of the works they consider most linked with their application (reprints and/or books).

The Jury is composed of members of the scientific committee of the Jean-Marie Delwart Foundation and of members of the Académie Royale des Sciences de Belgique.

The Prize will be awarded in December 2003 at the occasion of the Public Session of the Académie Royale des Sciences de Belgique.

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